

Neuregulin Protein Regulation of Synaptic Proteins

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Cross-Reference to Related Applications

This application claims priority to PCT Application No. US2004/009499, filed on  
5 March 26, 2004, which claims priority to U.S. provisional application number 60/457,321  
filed March 26, 2003, both of which are hereby incorporated by reference in their  
entirety.

Field of the Invention

10 The invention relates generally to control systems and pharmaceuticals for  
controlling synapse functioning and more specifically to methods, and materials for  
finding and using pharmaceuticals that alter PSD-95 and other components of synapse  
functioning.

Background

15 The transmembrane protein Neuregulin-1 ("Nrg-1") originally was identified as a  
44-kD glycoprotein that interacts with the NEU/ERBB2 receptor tyrosine kinase to  
increase phosphorylation of the glycoprotein's tyrosine residues. At least 15 isoforms can  
be produced from the Nrg1 gene by alternative splicing. These isoforms are tissue-  
20 specifically expressed and differ significantly in their structures. The major expressed  
form in the nervous system contains a highly conserved intracellular domain that has  
three splicing isoforms that are named the A, B, and C forms. The longest A form likely  
is the major isoform expressed in the nervous system. In the extracellular domains of all  
Nrg isoforms contain an epidermal growth factor-like (EGF-like) sequence. The  
25 receptors for Nrg-1 isoforms are the ERBB family of tyrosine kinase transmembrane  
receptors. Through interaction with ERBB receptors, Nrg-1 isoforms induce the growth  
and differentiation of epithelial, neuronal, glial, and other types of cells.

Recent research results suggest that the cytoplasmic portion of Nrg has a role in  
apoptosis, as reported by Grimm et al. in "Neu Differentiation Factor (NDF), a Dominant  
30 Oncogene, Causes Apoptosis In Vitro and In Vivo" J. Exp. Med., 188: No. 8 1535-1539  
(1998). Furthermore, a basic 157 amino acid sequence common to the cytoplasmic tails  
of all transmembrane neuregulin isoforms has been identified that interacts with LIMK1.

Presumably this interaction causes phosphorylation of the cytoplasmic portion of Nrg. The role of a 217 amino acid portion remains unknown, however.

Much research has focused on the Nrg protein because the protein may be involved in synaptic formation and maintenance, which is basic to proper brain functioning. However, no clear idea has emerged for how this protein is involved in synapse functioning. Any new information regarding this system should result in new treatment modalities for nerve function related diseases, as well as chronic diseases of aging.

10 Summary of the invention

One embodiment of the invention is a drug discovery method for identifying a compound (or compounds) that modulates the induction of PSD-95 by the Nrg-1/Eos signaling pathway comprising contacting one or more test compounds with the cytoplasmic domain of Nrg (Nrg-ICD) or a portion thereof, wherein the Nrg-ICD or portion thereof is encoded by a nucleic acid that hybridizes to a nucleic acid having SEQ ID NO: 1 in 5x SSC at 42° C, and identifying the binding between the one or more test compounds and Nrg-ICD or a portion thereof.

Another embodiment is a drug discovery method for identifying a compound that modulates binding of Nrg-ICD with a binding site of Eos, comprising:

- 20 (i) contacting one or more test compounds with Nrg-ICD or a portion thereof and with at least one binding site of Eos, wherein the Nrg-ICD is encoded by a nucleic acid that hybridizes to a nucleic acid having SEQ ID NO: 1 in 5x SSC at 42° C;
- (ii) contacting Nrg-ICD or a portion thereof with the at least one binding site of Eos in the absence of the one or more test compounds; and
- 25 (iii) identifying a difference in binding between Nrg-ICD or a portion thereof and with the at least one binding site of Eos between the contacting of (i) and the contacting of (ii).

Yet another embodiment is a drug discovery method for identifying a compound that modulates translocation of Nrg-ICD into a cell nucleus, comprising contacting a cell with one or more test compounds; and detecting movement of Nrg-ICD from the cell cytoplasm into the cell nucleus. Yet another embodiment is A method for identifying a compound that promotes or inhibits translocation of Nrg-ICD across the nuclear

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membrane of a cell, comprising: transgenically expressing in cells, a polypeptide complex comprising a nuclear localization sequence of Nrg-ICD and a regulatory marker, wherein the localization sequence of Nrg-ICD is at least 90% homogeneous with a portion that exceeds 20 amino acids of SEQ ID NO: 2 and the regulatory marker influences the  
5 expression of a gene when present within the nucleus of the cell; and contacting the cells with test compounds and determining whether a test compound affects translocation of Nrg-ICD across the nuclear membrane of the cell.

Still another embodiment is a method for identifying a compound that modulates the proteolysis of Nrg-1 to form Nrg-ICD, comprising incubating a cellular membrane  
10 form of Nrg-1 in the presence of the compound, and detecting the formation of a carboxylic end portion of Nrg-1 that is less than 60 kilodaltons in size. Yet another embodiment is a method for identifying a compound that modulates gene activity by binding to an Ikaous 1/2 sequence, comprising providing transgenic cells that contain a reporter gene operably coupled to a promoter that comprises Ikaous 1/2 sequence;  
15 contacting the cells with one or more test substances; and  
detecting the induction of the reporter gene in response to one or more test substances.

Yet another embodiment is a fusion polypeptide of a pharmaceutically active compound discovered by a method as described herein, comprising a first polypeptide portion of between 8 and 50 amino acids long that exhibits binding to Nrg-ICD or Eos  
20 and a second polypeptide portion comprising a transporter moiety of between 10 and 20 amino acids long. Yet another embodiment is a method for enhancing learning in an animal, comprising providing to the animal a compound that modulates the formation or translocation of Nrg-ICD into the nucleus of a nerve cell, wherein the compound is a fusion compound as described herein. Yet another is a method for preventing neuronal  
25 excitotoxicity in an animal, comprising providing to the animal a pharmaceutical that attenuates the nuclear signaling pathway of Nrg-1. Yet another embodiment is a transgenic animal with enhanced learning capability, produced by the process of stably incorporating an exogenous Nrg-1 gene into the animal and expressing the gene. Yet another embodiment is an isolated protein complex, comprising primarily of Ng-ICD and  
30 Eos. Yet another embodiment is a vector that comprises a gene encoding Nrg-ICD and a gene encoding Eos. In an embodiment, the Nrg-1 has a sequence selected from known

Nrg-1 sequences, including for example, the sequences comprising approximately 15 distinct structurally related isoforms (Lemke, Mol. Cell. Neurosci. 7:247-262, 1996 and Peles and Yarden, BioEssays 15:815-824, 1993), isoforms of *NRG-1* such as Neu Differentiation Factor (NDF; Peles et al., Cell 69, 205-216, 1992 and Wen et al., Cell 69, 559-572, 1992), Heregulin (HRG; Holmes et al., Science 256:1205-1210, 1992), Acetylcholine Receptor Inducing Activity (ARIA; Falls et al., Cell 72:801-815, 1993), and the glial growth factors GGF1, GGF2, and GGF3 (Marchionni et al. Nature 362:312-8, 1993). The contents of each cited reference, and particularly the genetic and amino acid sequences are specifically incorporated by reference.

Other embodiments readily will be appreciated by a skilled artisan upon reading the disclosure and the details in the references cited herein.

#### Figures

Figure 1 shows the amino acid sequence (SEQ ID NO: 1) of a human Nrg-1-ICD protein.

Figure 2 shows a nucleic acid sequence (SEQ ID NO: 2) that encodes the amino acid sequence of Figure 1 and that includes an open reading frame for this sequence.

Figure 3 shows two representative nuclear localization sequences, (SEQ ID NO: 3, and SEQ ID NO:4).

Figure 4 shows two alternative binding site sequences in Nrg-ICD for Eos, (SEQ ID NO: 5 and SEQ ID NO: 6).

Figure 5 shows a human Eos DNA binding domain (SEQ ID NO: 7).

Figure 6 shows a human Eos domain for binding to Nrg-ICD (SEQ ID NO: 8).

Figure 7 shows three peptides that block Nrg-ICD/Eos signaling pathways in vivo (SEQ ID NO: 9, SEQ ID NO: 10, and SEQ ID NO:11.)

Figure 8 depicts data showing the upregulation of PSD-95 and nuclear Nrg-ICD after synaptic activation. (a) Top, organ of Corti removed immediately after the sound stimulation. Bottom, organ of Corti removed 12 h after sound stimulation. Nuclei, labeled by bisbenzimidazole, were used to identify the inner (IHC) and outer hair cell (OHC) layers. PSD-95 immunofluorescence images were obtained 2 mm below the nuclei panels to localize PSD-95 immunoreactivity in SGN terminals. Images from normal transmission light (green) were added in the overlay panels. (b) Total cell extracts from four spiral

ganglia removed immediately (control) or 6 h after the sound stimulation were separated and subjected to western blotting with anti-PSD-95 and later anti-homer or anti-SV2. (c) The intensity of PSD-95 immunoreactivity on the western blots was quantified from three independent experiments and compared between the control and 6 h conditions after  
5 intensive sound stimulation (110 dB for 5 min). \*  $P < 0.01$  as compared to control (s.d.). (d) Three splicing isoforms of Nrg-ICD. (e) Total SGN extracts were subjected to anti-Nrg-ICD western blotting immediately (control) or 30 min after sound stimulation (2 min/86 dB). (f) Cytoplasmic and nuclear fractions isolated from spiral ganglia were subjected to anti-Nrg-ICD western blotting. The blots were then reprobated with antibodies  
10 to histone H1 and eIF5. (g) The intensity of Nrg-ICD immunoreactivity on the blot was quantified from three independent experiments. \*  $P < 0.05$  as compared to control (s.d.).

Figure 9 depicts data showing the activation of the PSD-95 promoter by the Nrg-ICD and Eos complex on Ikaros sites. (a) Nrg-ICD does not bind to the SP1 probe in EMSA. (b) Nrg-ICD binds to the Ikaros binding site. (c) A dual luciferase assay  
15 demonstrating possible regulations of the PSD-95 promoter by Nrg-1 and Eos. 293T cells were cotransfected with a luciferase reporter gene under the control of PSD-95 promoter, a Renilla luciferase reporter vector (to normalize for transfection efficiency) and other expression vectors as indicated. Data are expressed relative to the control (s.d.). (d) Effects of progressive 5' deletions on Ikaros sites in the PSD-95 promoter, as detected by  
20 a luciferase assay. 293T cells were cotransfected with each of three promoter constructs (pGL3a, containing all five normal Ikaros sites; pGL3b, containing one; and pGL3c, containing none), a Renilla luciferase reporter vector and vectors expressing Eos and Nrg-1. Measurements from all three groups were normalized to the control (transfected with pGL3c only) and reported as fold increase (s.d.). (e) Effects of site-directed mutagenesis  
25 on Ikaros sites in the PSD-95 promoter, as detected by luciferase assay. Each of three promoter vectors (pGL3a, containing all five normal Ikaros sites; pGL3d, containing only one; and pGL3e, with none) was introduced into E16 cortical neurons. Values shown are s.d.

Figure 10 depicts data showing that Nrg-ICD-Eos nuclear signaling is mainly  
30 independent of  $\gamma$ -secretase. (a) Coimmunoprecipitation of Nrg-ICD and Eos. Left, cell extracts from 293T cells overexpressing Flag-Eos in the presence or absence of Nrg-1

were immunoprecipitated with an anti-Nrg-ICD and then immunoblotted with anti-Flag. Right, for the reverse coimmunoprecipitation, cell extracts from 293T cells overexpressing Nrg-1 in the presence or absence of Flag-Eos were immunoprecipitated with anti-Flag and then immunoblotted with Nrg-ICD (right). (b) Binding of the Nrg-1/Eos complex to the Ikaros site. Nuclear extracts from 293T cells transfected with Nrg-1 and Flag-Eos were mixed with a normal (lane 1, 2, 4–6) or a mutated Ikaros probe (lane 3, 7, 8). (c) Three Nrg-1 fragments mentioned in d,e. (d) Identified Nrg-ICD fragments responsible for PSD-95 upregulation. 293T cells (open bars) or PS1- PS2- cells (filled bars) were cotransfected with a luciferase reporter gene under the control of the PSD-95 promoter, a Renilla luciferase reporter vector and other expression vectors as indicated. Data are expressed relative to the control group ( s.d.). (e) Left, cell extracts from cells expressing Nrg-1 (tagged with HA at its C terminus) with either wild-type PS1 (PSwt) or loss-of-function mutant variants of PS1 (dAsp) were analyzed by anti-HA western blotting. Right, cell extracts from 293T cells expressing Nrg-1 and treated with -secretase inhibitors, at concentrations as indicated, were analyzed by anti-HA western blotting.

Figure 11 depicts data showing the regulation of endogenous PSD-95 by Nrg-1 and Eos. (a) ChIPs were performed on 293T cells transfected with Eos, Nrg-1 or Nrg-1 and Eos by anti-Nrg-ICD or Flag antibodies. Associated DNA was analyzed by PCR using human PSD-95 promoter specific primers. (b) Whole cell extracts from 293T cells were used in a subjected to anti-Nrg-ICD (top) or anti-Flag (bottom) western blotting. (c) Cell extracts from 293T cells transfected with mammalian expression vectors expressing either Nrg-1/Eos, Nrg-1, Eos or Pegasus (as indicated) were subjected to western blotting with anti-PSD-95, anti-Flag or anti-Nrg-ICD. (d–g) Confocal images were collected from 293T cells (d), PS1- PS2- cells (e) or neurons (f,g) transfected with the vectors indicated at left, after immunostaining with the antibodies indicated above.

Figure 12 depicts the synaptic activity dependent activation of the PSD-95 promoter by Nrg-ICD. (a) Binding of the Ikaros site in SGNs 1 or 30 min after sound stimulation. (b) ChIPs with anti-calretinin or anti-Nrg-ICD were performed on nuclear extracts from SGNs exposed to the sound. Associated DNA was analyzed by PCR using primers specific for the mouse PSD-95 promoter. (c) Decrease in PSD-95 promoter activity resulting from interfering Nrg-ICD/Eos signaling. E16 cortical neurons were

cotransfected with a luciferase reporter gene under the control of the PSD-95 promoter and 2 mg of other expression vectors as indicated. To block the interaction between Nrg-1 and Eos, 12 mg (6 ) or 120 mg (60 ) Eos-C were used in the indicated groups, and the total DNA concentration was equalized among all groups by the control pcDNA3  
5 plasmid. For half of the groups, 50 mM KCl was applied to mimic neuronal activity 24 h after cotransfection. The luciferase activity data from the readout were directly used for the graph ( s.d.).

Figure 13 depicts the visualization of the nuclear translocation of Nrg-1-ICD–GFP in living cells. (A) The schematic illustration at the top shows the NRG-1βa-GFP  
10 chimeric used in this work. ECD, extracellular domain; TM, transmembrane domain; NLS1, the putative nuclear localization sequence (B); ICD, intracellular domain; GFP, green fluorescent protein. Intracellular movement of NRG-1βa-GFP was followed in live cells (HEK 293 cells) by two-photon microscopy. Images (1 μm from the middle of the nucleus) were collected at various intervals (min) after treatment with soluble erbB2 +  
15 erbB4. The arrows in the enlargement point out puncta of Nrg-1-GFP that have entered the nucleus. (B) HEK293 cells were transfected with plasmids encoding either an intact Nrg-1-ICD fused to GFP (top) or an Nrg-1-ICD lacking the putative NLS fused to GFP (bottom). The subcellular localization of the fusion proteins was followed by conventional fluorescence microscopy. At left are phase images. In the middle, the green  
20 fluorescence signal is shown (arrows point to positive cells), and at the right, both green fluorescence and DAPI staining are shown. (C) Cytoplasmic (Cyto) and nuclear (Nuc) fractions were prepared from mock transfected or NRG-1βa-HA (HA, influenza virus hemagglutinin-derived epitope added to COOH terminus of Nrg-1)–transfected HEK293T cells. Proteins were analyzed by immunoblotting using antibodies recognizing  
25 the HA epitope. NRG-1βa-HA– transfected cells were treated for 15 min with erbB2 (32 μg/ml) or erbB2:4 (32 μg/ml). In addition to a doublet of nonspecific bands, proteins of >100 kD (full-length and aggregated NRG-1βa) and 50 kD (ICD-HA) were detected. The 50-kD band enriched in the nuclear fraction was only seen in cells treated with soluble erbB2:B4.

30 Figure 14 depicts a schematic illustrating bidirectional signaling by transmembrane Nrg-1. Both forward and back signaling result from interactions between

erbB receptors (blue) and membrane-tethered Nrg-1 (green and red). Interaction (steps 1 and 2) results in activation of erbB receptor tyrosine kinases and subsequent induction of target genes (step 6) in erbB-expressing cells, as well as intramembranous (and possibly extracellular) cleavage of Nrg-1 (steps 3 and 4). The released Nrg-1-ICD (red) translocates from the neurites to cell bodies (step 5a), and then to the nucleus, possibly with other proteins (step 5b), where it regulates target gene expression (step 5c).

#### Detailed Description

The inventors discovered that the cytoplasmic portion of Nrg-1 enters the cell nucleus and regulates PSD-95 and other synaptic scaffolding protein transcriptions. Furthermore, the nuclear translocation of the Nrg-1 intracellular domain was found to be increased by neuronal activity. Moreover, this increase was shown to be accompanied by enhancement of PSD-95 protein through binding between the intracellular domain of Nrg-ICD and a zinc-finger protein of the transcription factor Eos, which was found to interact with the PSD-95 promoter and induce endogenous PSD-95 expression in both non-neuronal and neuronal cells. These discoveries were translated into several technologies of significant importance to biomolecular and cellular mechanisms of learning, memory, and other neurological processes.

#### **A variety of new technologies**

From these findings, new drug development technologies are invented for discovering pharmaceutically active compounds that can improve neuronal adaptation and/or alleviate neuronal diseases. Such compounds may be used for improved learning, improved memory, and alleviation of diseases associated with neuronal malfunctioning. Additional discoveries based on these findings include the ability to engineer human and other animals with improved memory and learning capacity.

The discoveries can be used in various ways to create new materials and new methods that impact neuronal functioning. For example, chemical and biomolecular intervention may, for example, occur at the level of Nrg-ICD processing at the cell membrane, transit of Nrg-ICD into the nucleus, interaction of Nrg-ICD with other protein in the nucleus, synthesis of Eos and use of Eos. In each case, assays can be formulated for discovery of drugs that modulate the process. Genetic modifications to the neuron



genome and other cell genomes may be carried out to effect changes at these points as well.

New sequences and localized portions of sequences have been discovered and found useful for many embodiments. In one group of embodiments, the gene promotion activity of Nrg-ICD is monitored, modified and/or otherwise exploited. The Nrg-ICD - Eos complex affects a specific promoter of the PSD-95 gene. That promoter may be coupled to another gene to allow regulation of another gene. Furthermore, increased amount of Nrg-ICD and, (optionally), increased amount of Eos protein may be produced by up-regulating their genes and/or by transgenic expression leading to more of these protein(s).

The mechanisms of protein-based interactions discovered and explained herein also are a rich source for biochemical intervention. For example, the specific interaction between Nrg-ICD and a region of the zinc-finger transcription factor, Eos may be monitored, modified or otherwise exploited. This specific binding leads to increased expression of the PSD-95 gene. Modification of this binding with a specific compound or protein that may be supplied from outside the cell, or that may be supplied from expression within the cell, can modulate PSD-95 gene activity, with concomitant effects on memory and learning.

The translocation of Nrg-ICD into the nucleus can be monitored or even controlled in embodiments. Nrg-ICD translocation can be measured, for example, by monitoring changes in the amount in the cytoplasm and/or the nucleus. Nrg-ICD can be expressed as a conjugate with a marker such as green fluorescent protein. Nrg-ICD also can be detected more indirectly by, for example, immunolabelling. Yet another useful method is to couple the presence of Nrg-ICD in the nucleus with expression of a reporter gene. For example, a cell may be transformed with a reporter gene coupled to the PSD-95 promoter or other promoters containing one or more Ikaros sites. Translocation of Nrg-ICD to the nucleus may be monitored by expression of the reporter. In one embodiment a non-neuronal cell system, wherein the PSD-95 gene is silent, is used so that the reporter gene promoter does not compete with an active PSD-95 gene promoter. In another embodiment a transgenic cell is used that also expresses an Nrg-ICD and/or the Eos gene.

In sum, embodiments of the invention provide the ability to independently control the Nrg-ICD and Eos genes, as well as their protein products at a variety of control points. For example, the Eos gene may be altered or its protein functioning interfered with to affect PSD-95 transcription. Peptides that include portions of the Eos binding sites, preferably at least 8, 9, 10, 15, 20, 30, 40 or even longer portions, may be provided extracellularly, or intracellularly transgenically to compete with Eos for binding to Nrg-ICD or to DNA (see SEQ ID NO: 8 and SEQ ID NO: 7, respectively). Similarly, peptides that include portions of the Nrg-ICD binding sites, preferably at least 8, 9, 10, 15, 20, 30, 40 or even longer portions, may be provided extracellularly, or intracellularly transgenically to compete with Nrg-ICD for binding to Eos and/or DNA (see SEQ ID NO: 5, SEQ ID NO: 6). In a desirable embodiment a nucleic acid that is similar (at least 80%, 90%, 95% or higher homology) to either the Eos gene or the Nrg-ICD gene is provided to affect the respective gene. In some embodiments, two or more factors are used simultaneously for stronger control of PSD-95. Pharmaceutically active compounds may be discovered by assaying binding to portions of the Eos gene, the Eos protein, the Nrg-1 gene, the Nrg-1 protein, and also by assaying biological activity of the two proteins in response to presence of test compounds.

I. DRUG DISCOVERY

(i) *Nrg-ICD processing at the cell membrane*

In an embodiment of the invention, phosphorylation of the cytoplasmic portion of Neuregulin-1 growth factor is monitored to determine the effects of compounds on this process. Any technique that detects the incorporation of phosphate into the Neuregulin-1 growth factor may be used. In an embodiment, an assay that measures phosphate incorporation into this membrane protein within a cell is coupled with the exposure of the cell to one or more test compounds. For a control, preferably the same condition is used in the absence of a test compound.

In a desirable embodiment the incorporation of phosphate is further coupled with another event such as proteolysis of the protein or translocation of a carboxyl terminal fragment created by proteolysis into the nucleus. In one embodiment, proteolysis or phosphate incorporation is coupled with the incorporation of a reporter substance such as

a fluorescent compound or atom and the reporter substance is used to monitor transit into the nucleus.

In another embodiment proteolytic processing of the cytoplasmic portion of the Neuregulin-1 growth factor is monitored to determine the effects of compounds on this process. Any technique that detects the formation of a smaller portion, such as a 53 kilodalton piece, or a 35 kilodalton piece may be used. For example, after incubation, cells may be lysed, water soluble (not membrane bound) molecules separated and the carboxyl terminal portion may be quantitated by immunoreaction with a suitable antibody conjugate. In a desirable embodiment the effect of a test compound on the proteolytic reaction may be tested by contacting the cells (or membrane fraction that contains the Neuregulin-1 growth factor) with test compounds. A control may be to contact cells with no compound, or with a compound that is not active in the system. Modulation of proteolysis may be determined by the effect of a compound on the amount of proteolytic product made.

Proteolytic processing converts the Nrg-1 protein to a smaller Nrg-1-CIP protein of about 35 kilodaltons, in normal conditions. The term "about" in this context means with the regular precision accompanying gel electrophoresis determinations of protein size. For example, a protein of 25 kilo Daltons sometimes can be detected as 35 kilo Daltons. And a protein of 45 kilo Daltons sometimes can be detected as 35 kilo Daltons. In other instances such as a disorder that leads to non-natural processing, another size such as about 53 kilo Daltons may be made.

*(ii) Translocation of Nrg-ICD to the nucleus*

In an embodiment, the translocation of a carboxylic acid portion of the protein that is created by proteolysis from the cytoplasm to the nucleus is monitored. Such monitoring provides much information regarding neural processing and is very useful for drug discovery assays. A large variety of techniques may be used to monitor translocation. Some of these techniques are useful for large numbers of samples and are particularly desirable for drug discovery. For example, see U.S. No. 6,416,959 (System for cell-based screening), which describes systems, methods, screens, reagents and kits for optical system analysis of cells to rapidly determine the distribution, environment, or activity of fluorescently labeled reporter molecules for large scale screening. Also see

U.S. No. 20020042366 (Method for treating inflammation), which describes a nuclear translocation assay that was used to identify transcription factor proteins that move from the cytoplasm to the nucleus by immunofluorescence. U.S. No. 20010041347 (System for cell-based screening) also teaches automated systems, methods, screens, and software that may be used to monitor movement of Nrg-ICD into the nucleus.

Yet another system that uses microbead-based test plates and test methods for fluorescence imaging systems is U.S. 20020098588, issued July 25, 2002. Other relevant techniques are known to the skilled artisan, such as for example that taught by Feng Yang et al. in *Analytical Biochemistry* 266: 167-173 1999 (highly efficient green fluorescent protein based kinase substrates) and by Javier Farinas et al. in *The Journal of Biological Chemistry* 274: 7603-7606 1999 (Receptor mediated targeting of fluorescent probes in living cells). Each of these references, and particularly the materials and methods referred to, are incorporated by reference in their entireties as space and time limitations prevent importing further detail from these references.

Detecting translocation into the nucleus is particularly useful for drug discovery assays. Typically, a cell is contacted with solution that contains one or more test substances and the amount of Nrg-ICD in cytoplasm, nucleus or both is determined to find out a relationship between a test substance and the amount of Nrg-ICD that moves into the nucleus. A positive correlation with a test compound (increase in amount found in the nucleus) may indicate an effect of the compound on generation of Nrg-ICD, release of Nrg-ICD, stability of Nrg-ICD translocation of Nrg-ICD or the like. Such positive correlation may reveal that the compound can stimulate a neurological process such as memory or learning, generally through the up regulation of PSD-95.

*(iii) Binding between Nrg-ICD and Eos to form a Nrg-ICD-Eos Complex*

In another embodiment binding between Nrg-ICD and human Eos is monitored. In one such embodiment the binding affinity is quantified and used to compare different Nrg-ICD's having different sequences. For example, a range of Nrg-ICD's from different animals and having different sequences are known. See, for example the sequence information provided in references cited herein. Despite the sequence differences, each Nrg-ICD that has at least some binding to human Eos has utility for drug test compound screening purposes. Preferably a sequence or sequence fragment is homologous enough

to Nrg-ICD such that the protein or peptide binds to Eos with an affinity of at least 5% of the affinity of human Nrg-ICD of 35 kilo Dalton size. More preferably a sequence or sequence fragment shares enough homology such that the sequence or sequence fragment has at least 10% of the affinity of human Nrg-ICD to the Eos. Yet more preferably the  
5 homology is closer and provides at least 25% or even at least 50% or the affinity of human Nrg ICD. Thus, the xenopus Nrg-ICD sequence can be used for the discovery of drugs that are active in humans.

Binding measurements can be carried out by a large number of procedures. For example equilibrium dialysis may be carried out. Bound from non-bound fractions may  
10 be separated by column chromatography, and bound from non-bound phases may be separated by regular solid phase separation techniques. Identification of bound complex likewise may be achieved by use of a reportable label or labels on the components. In one high throughput multi-sample assay, fluorescence resonance energy transfer is used to detect when a first fluorescent probe on Nrg ICD or fragment thereof, is close to a  
15 second probe on Eos.

In an embodiment the binding between Nrg-ICD and Eos is probed in the presence of one or more test compounds as a drug screening assay. Generally, the two molecules are incubated in the presence of one more test compounds to determine whether the test compound facilitates or inhibits binding. A compound that affects  
20 binding may be selected as a lead compound for affecting a neurological system such as memory, learning, hyper stimulation and the like.

In another embodiment, an assortment of genes are prepared that encode modifications of the Nrg-ICD sequence or fragment of the Nrg-ICD sequence. A combinatorial library selection procedure is used to select one or more nucleic acid  
25 sequences that bind with greater binding affinity to Eos. Such techniques are known, as exemplified in U.S. Nos. 5,807,683 (Combinatorial libraries and methods for their use); 5,738,996 (Combinational library composition and method); 6,518,065 (Methods for generating polynucleotides having desired characteristics by iterative selection and recombination); 6,172,197 (Methods for producing members of specific binding pairs);  
30 6,132,970 (Methods of shuffling polynucleotides); and 6,007,988 (Binding proteins for recognition of DNA), the contents of which are specifically incorporated by reference in their entireties. Using one of these or other known procedures, an improved Nrg-ICD

sequence may be designed having greater affinity for Eos. Alternatively, an improved Eos may be designed having greater affinity for Nrg-ICD. In another embodiment one or more improved proteins is further tested to determine its effect on transcription of the PSD-95 protein.

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*(iv) Binding between Nrg-ICD-Eos Complex and a Gene Promoter*

It was discovered that an Nrg-ICD-Eos complex up regulates the PSD-95 gene through interaction with the promoter of this gene. In an embodiment this binding is assayed and used to detect a compound having pharmaceutical activity. One or more test  
10 compounds are incubated in the presence of a cell or cell free system that comprises the Nrg-ICD - Eos complex and the PSD-95 gene promoter. Binding between the complex and the promoter is detected and used to determine whether one or more test compounds may have pharmaceutical activity. In a particularly desirable embodiment the PSD-95 promoter is operably linked, and preferably cis-linked to a reporter gene that creates a  
15 detectable signal after the gene's transcription.

*(v) Cell culture screening of compounds*

Compounds may be screened by looking at their effects on one or more aspects of the PSD-95 control system in cells. For example, PSD-95 synthesis, or capability to regulate PSD-95 synthesis, may be monitored in transgenic cells that utilize a reporter  
20 gene coupled to one or more Ikaros sites. Preferably, the one or more Ikaros sites comprise a specific Ikaros sequence of the PSD-95 promoter. Upon increased Nrg-ICD, increased translocation, or increased binding of Nrg-ICD-Eos to DNA etc., the transcription of a reporter gene that has this promoter will be increased, causing increased amount of the reporter gene product. The increased amount may be determined optically  
25 or by any other relevant method.

Large scale cell detection systems may be used in a wide variety of configurations. Typically, individual cells, or groups of cells are cultured and exposed to one or more test substances. An index of cellular health or some other biochemical or biological response is monitored and correlated with the presence of specific compounds.

30 For example, cell death may be detected fluoroscopically, as for example taught in U.S. No. 5,534,416 (Fluorescent viability assay using cyclic-substituted unsymmetrical cyanine dyes) and other references cited therein. A variety of cell manipulation and assay

technologies are available, such as those promoted by Axiom Technologies, Inc. of San Diego (see U.S. 5,804,436); Caliper Technologies Corporation of Palo Alto (see U.S. 5,942,443; 6,001,231; 6,046,056; 6,132,685; 6,149,787; and 6,529,835). A variety of cell imaging systems are known and particularly useful, as exemplified by U.S. 5,627,643 (Method and detector for separation processes) and the citations therein. Each of these cited patents specifically is incorporated by reference in its entirety, and more specifically, the materials and methods for manipulating cells and detecting changes in the cells, for application to embodiments of the invention. Due to space and time limitations, further details from these 9 patents and further patents cited therein are not described further.

(vi) *Screening compounds in vivo*

As an initial screen, and also as a later screen following mass screening of large numbers of compounds, an *in vivo* test assay is particularly desirable. In one such embodiment a test chemical is linked with a short peptide that facilitates cell entry. The conjugate is introduced into an animal body by, for example, injection intraperitoneally, into the brain or other lumen. After waiting a period of time for cell entry and effect a response is measured. The waiting time typically may be from about 1 minute to 10 days, preferably from 15 minutes to 2 days, and more preferably from about 30 minutes to one day. The effect of the conjugate may be determined on the peripheral nervous system such as dorsal ganglion or the central nervous system. Any index of biological, biochemical, physiological, or behavioral response may be determined. For example, PSD-95 induction in dorsal ganglion may be determined after pain stimulation; PSD-95 induction in spiral ganglia may be determined after noise stimulation; and PSD-95 induction in the hippocampus may be determined after learning. Other assays may be used, such as measuring neuronal cell death after environmental stimulation, to show protection against over stimulation.

In a favorable embodiment, a peptide having a sequence SEQ ID NO: 9; SEQ ID NO: 10; SEQ ID NO: 11, or a derivative thereof is used *in vivo* for altering PSD-95.

These peptides comprise a cellular HIV-1 permeator moiety. A variety of sequences may be used for modifying PSD-95 through cell entry in this fashion. Other sequences as described herein may be used. Conservative amino acid substitutions corresponding up to

1%, 2%, 5%, 10%, 15%, 20% or even more of a peptide sequence may be used without completely abolishing activity and are desirable.

## II. Therapy / Prophylaxis

### 5 (i) *Increase Neuregulin-1 and/or Eos for Improved Memory/Learning*

In an embodiment of the invention, the amount of precursor to Nrg-ICD in a cell is increased. This increase leads, directly or indirectly to improved memory and/or learning performance of an animal that contains nerve cells with the increased precursor. Without wishing to be bound by any one theory for this embodiment of the invention it is  
10 believed that at least in some instances, memory and/or learning is limited by the amount of PSD-95 in synapse generating cells. The amount of PSD-95 can be increased by upregulating PSD transcription. The increased PSD-95 provides faster and/or greater strengthening of synaptic transmission in response to signals that transit the synapse junction. Because of the greater response, less stimuli is needed to effect a change in the  
15 synapse. The lesser stimuli needed means that less effort is required to remember or to learn something.

In an analogous way, the amount of Eos available can be modified as well. The increase in Eos can lead to improved memory, and/or learning performance. In some individuals, a limitation in the amount or quality of Eos is rectified by introducing more  
20 of this protein into cells, either endogenously (by protein synthesis within the cell) or (less preferably) exogenously.

A skilled molecular biologist can increase Neuregulin-1 and/or Eos several ways that will be apparent to a reader. For example, foreign Neuregulin and/or Eos gene(s) may be added transgenically. Homologous recombination also may be carried out by  
25 incorporating a constitutive or other strong promoter or enhancer to upregulate a native (existing in the cell) Neuregulin or Eos gene. A variety of genetic transformation techniques and vectors may be used for this purpose, as described below.

In another embodiment, one or more extracellular effectors cause proteolysis of Nrg-1 to form Nrg-ICD. A drug screening assay may be carried out wherein detection of  
30 ICD formation (transport into the nucleus, upregulation of PSD-95 or other gene such as a reporter gene) is monitored in cells. Cells are contacted with different test compounds and the effects are correlated to determine whether a compound affects activity.



In a desirable embodiment one or more extra Eos genes are added (or up regulated) and one or more Neuregulin-1 genes are added (or up regulated) to further facilitate an increase in Nrg-ICD - Eos complex for up regulating the PSD-95 gene. In yet another embodiment one or more PSD-95 genes also are added to help increase the amount of PSD-95 expression. Accordingly, embodiments of the invention include vectors that comprise one or more such genetic elements and also cells and transgenic animals that contain one more such genetic elements. A desirable embodiment provides a vector that comprises both a PSD-95 gene and a Neuregulin-1 gene.

A particularly desirable embodiment is a method for improving nerve cell function comprising adding one or more genes as detailed herein via a vector to cells that contain excitatory membrane or that develop into cells that contain excitatory membranes. The gene(s) preferably are added via a vector such as a viral vector. The cells may be cultured cells and later added to an animal. Alternatively, the cells may exist in vivo and the method provides improved functioning of a pre-existing nervous system.

*(ii) Decrease Neuregulin-1 for Treatment of Ischemic Brain Damage, Overstimulation*

In some embodiments, materials and methods are used to down-regulate PSD-95. As described, for example, by Aarts et al. in Science, Vol. 298 pp. 846-850 (2002) down regulation or targeting of PSD-95 protein represents an alternative therapy for diseases that involve excitotoxicity and ischemic brain damage. Embodiments of the invention can inhibit the synthesis of PSD-95. Interference of the synthesis or functioning of Nrg-1 or of Nrg-ICD may be used to down regulate PSD-95 for desirable clinical effects.

Most desirably, materials and methods may be used to prevent neuronal death after damage, such as noise in the spiral ganglion, injury in the spinal cord and brain, or chronic pain from a dorsal root ganglion or spinal cord. In an embodiment, a substance that inhibits the formation of Nrg-ICD; translocation of Nrg-ICD into the nucleus; binding of Nrg-ICD to Eos; formation of Eos; and/or binding of Nrg-ICD-Eos to the PSD-95 promoter is used prior to, immediately after or at longer times after an event leading to neuronal stress. In an embodiment, the substance is administered to a person prophylactically at least 1 second, 1 minute, 5 minutes, 1 hour, 4 hours, a day, or even a longer period prior to an expected exposure to neuronal stress, such as high noise level, impact injury (such as boxing) to the brain, or other stress. In another embodiment the

substance is administered long term (at least once daily, weekly or monthly) to counteract chronic pain. In an embodiment the substance is administered by injection and in another embodiment the substance is administered orally. The effect of administration is to desensitize neurons to overstimulation (or to remove oversensitivity to normal stimulation) at affected neurons, and thereby minimize cell death.

*(iii) Peptides for down-regulating the PSD-95 induction system*

In some disease processes and physiological states an excess of stimulation and/or other perturbations in the overall biochemical system of PSD-95 induction by Nrg-ICD is alleviated by one or more peptide agents. In an embodiment, the peptide corresponds to a continuous sequence of at least 8 amino acids selected from any of SEQ ID NO: 1, 3, 4, 5, 6, 7, 8, 9, 10, and 11. In a desirable embodiment, the peptide is fused to a cellular entry factor such as the HIV-1 Tat residues 38 to 48 outside the transduction domain, as for example, described in Aarts et al. Science 298: 846-850 (2002) and Mann et al. EMBO J. 10: 1733 (1991). In a particularly desirable embodiment the peptide comprises a sequence SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO:11 (see Figure 7) or a modification of such sequence.

*(iv) Increase translocation of Nrg-ICD for enhanced PSD-95 induction*

In another desirable embodiment, a modified Nrg-ICD is created that translocates into the nucleus more readily. In one embodiment, an extra segment of translocation sequence is added to the carboxyl terminal region, and desirably to the carboxy terminal end of the Neuregulin-1 gene. Examples of such translocation sequences are SEQ ID NO: 3 and SEQ ID NO: 4 provided in Figure 3. A result of the increased translocation is increased PSD-95 synthesis, leading to improved synapse performance in many instances. In an embodiment, either sequence is added to another protein to direct translocation of that protein into the nucleus. In yet another embodiment, the two sequences are used in their relative position along the protein to be transcribed to direct a protein into the nucleus. In another embodiment two or more copies of a translocation sequence are added to a protein or to a gene encoding a protein.

(v) *Increase proteolysis of Neuregulin-1 for increased Nrg-ICD production*

In another desirable embodiment, a modified Neuregulin-1 is produced that has at least one extra proteolytic site for enhanced sensitivity to proteolysis. In an embodiment, the site comprises one of the sequences: QRKK, QRKL, SPHS and SPHE (SEQ ID NOS: 12-15). In one embodiment one extra site containing one or more of these sequences is added. In another embodiment one, two, three, four, five or more amino acids such as glycine, serine, threonine or alanine are added between proteolysis sites to move a proteolysis spot away from the transmembrane region and from other sites.

In an embodiment two, three, four or even more proteolysis sites that comprise at least 6 continuous amino acids centered at the middle of regions that contain QRKK, QRKL, SPHS, or SPHE (SEQ ID NOS: 12-15), are added adjacent to (on the carboxyl terminal side or on the amino terminal side of) the naturally occurring site. By "middle" is meant the point between the second and third amino acid of each group of four. In an embodiment the added site(s) are separated from adjacent sites by one, two, three, four, five, six, seven, eight, nine, ten or even more amino acid spacers. In another embodiment at least 6, 7, 8, 9 or even more than 10 continuous amino acids centered at the middle are added. Preferably a spacer comprises one or more small neutral amino acids such as glycine, alanine, serine, threonine, and the like.

(vi) *Increase Eos binding sites for improved complex formation*

In another desirable embodiment, a modified Neuregulin-1 is produced that has more than one copy of the Eos binding site at its carboxyl terminus region. In an embodiment two, three, four or even more Eos binding sites that comprise at least 15, 20, 25, 30 or more continuous amino acids from SEQ ID NO: 5 and/or SEQ ID NO: 6 are added adjacent to (on the carboxyl terminal side or on the amino terminal side of) the naturally occurring site. In an embodiment the added site(s) are separated from adjacent sites by one, two, three, four, five, six, seven, eight, nine, ten or even more amino acid spacers. In another embodiment at least 35, 40, 50, 9 or even 60 continuous amino acids of the Eos binding site are added. Preferably a spacer comprises one or more small neutral amino acids such as glycine, alanine, serine, threonine, and the like.

III. Materials for Embodiments of the Invention

A wide variety of cell types, animals, vectors, nucleic acids, and proteins may be used for embodiments of the invention. In particular, for drug discovery assays, a comparative test result most often is obtained to determine whether a test compound has activity. Accordingly, such tests do not require complete and exact human sequences of protein or nucleic acid. In an embodiment, an Nrg-ICD protein (or Eos protein) that binds at least 1%, 2%, 5%, 10%, 20%, or at least 50% (determined as an affinity constant) as well to its cognate binding site compared to an exact and complete human sequence may be used for drug discovery tests. As seen in cited references, for example, the cytoplasmic domain sequences obtained from different species typically have more than 70%, 80%, 85%, 87%, 90%, 95%, 97% or even greater homology. Each of these sequences displays measurable activity in one or more assays or tests described herein. Furthermore, other variations of sequences also lead to protein having desirable activity.

*Cells*

Many embodiments utilize intact cell synthesis of Neuregulin-1 and the modulation of PSD-95 protein. Many of the desirable cells are obtained from a kinder or brain cell lineage. In many instances, a protease in the cell acts upon neuregulin-1 to generate Nrg-ICD on the cytoplasmic side of the cell membrane. The Nrg-ICD enters the cell nucleus and upregulates PSD-95 or another gene. For assays and other methods that utilize one or more of these events, it is particularly preferred to use a cell that normally expresses Eos and preferably PSD-95 as well or another regulated protein having a promoter that responds to the Nrg-ICD-Eos complex. Preferred cell lines are 293T and 293S. Other cell lines that have endogenous Eos are particularly desirable because only Nrg-1 needs to be expressed in certain embodiments that utilize these cells.

*Transgenic Animals*

In embodiments of the invention one or more genes are introduced into and stably expressed in an animal such as a human, mouse, canine, monkey, rat, ovine, bovine or other animal. In desirable embodiments a Neuregulin-1 gene such as the human gene is introduced into such animal and results in improved synaptic functioning. In another

embodiment a normal human gene is introduced into a human cell that contains a gene that is genetically abnormal, and improves cellular function. In another embodiment, both Neuregulin-1 and PSD-95 genes are introduced. In yet another embodiment, both Neuregulin-1 and Eos genes are introduced. In yet another embodiment, a polypeptide sequence as described herein is expressed transgenically. A transgenic animal produced thereby can be used, for example, as a test system for evaluating compounds for pharmaceutical effect.

### *Vectors*

A variety of vectors may be use for transfer of genetic information into a cell according to embodiments of the invention. The term "vector" as used herein refers to a construction comprised of genetic material designed to direct transformation of a targeted cell. A vector contains multiple genetic material, preferably contiguous fragments of DNA or RNA, e.g., DNA derived from a plasmid, cosmid, phasmid or bacteriophage or synthesized by chemical or enzymatic means, positionally and sequentially oriented with other necessary elements such that the nucleic acid can be transcribed and when necessary translated in the transfected cells. The vector can contain one or more unique restriction sites for this purpose, and may be capable of autonomous replication in a defined host or organism such that the cloned sequence is reproduced. The vector may have a linear, circular, or supercoiled configuration and may be complexed with other vectors or other materials for certain purposes. The components of a vector can include but are not limited to a DNA molecule incorporating: (1) a sequence encoding a therapeutic or desired product; and (2) regulatory elements for transcription, translation, RNA stability and replication.

In embodiments, a preferred vector comprises the following elements linked sequentially at an appropriate distance to allow functional expression: a promoter, a 5' mRNA leader sequence, a translation initiation site, a nucleic acid cassette containing the sequence to be expressed, a 3' mRNA untranslated region, and a polyadenylation signal sequence. As used herein the term "expression vector" refers to a DNA vector that contains all of the information necessary to produce a recombinant protein in a heterologous cell.

In addition, the term "vector" as used herein can also include viral vectors,

although non-viral vectors are preferred. A "viral vector" in this sense is one that is physically incorporated in a viral particle by the inclusion of a portion of a viral genome within the vector, e.g., a packaging signal, and is not merely DNA or a located gene taken from a portion of a viral nucleic acid. Thus, while a portion of a viral genome can be  
5 present in a vector of the present invention, that portion does not cause incorporation of the vector into a viral particle and thus is unable to produce an infective viral particle.

A vector as used herein can also include DNA sequence elements, which enable extra-chromosomal (episomal) replication of the DNA. Vectors capable of episomal replication are maintained as extra-chromosomal molecules and can replicate. These  
10 vectors are not eliminated by simple degradation but continue to be copied. These elements may be derived from a viral or mammalian genome. These provide prolonged or "persistent" expression as described below.

The vector can be used to provide expression of a nucleic acid sequence in tissue. In the present invention this expression preferably is enhanced by providing stability to an  
15 mRNA transcript from the nucleic acid sequence and/or secretion of the therapeutic protein. Expression includes the efficient transcription of an inserted gene or nucleic acid sequence within the vector. Expression products may be proteins including but not limited to pure protein (polypeptide), glycoprotein, lipoprotein, phosphoprotein, or nucleoprotein. Expression products may also be RNA. The nucleic acid sequence is  
20 contained in a nucleic acid cassette. Expression of the nucleic acid can be continuous or controlled by endogenous or exogenous stimuli.

The term "persistent expression" as used herein refers to introduction of genes into the cell together with genetic elements which enable episomal (i.e., extrachromosomal) replication. This can lead to apparently stable transformation of the cell without the  
25 integration of the novel genetic material into the chromosome of the host cell.

"Stable expression" as used herein relates to the integration of genetic material into chromosomes of the targeted cell where it becomes a permanent component of the genetic material in that cell. Gene expression after stable integration can permanently alter the characteristics of the cell and its progeny arising by replication leading to stable  
30 transformation.

*Proteins*

The term "Neuregulin-1" and "Nrg-1" are used interchangeably herein and refer to a protein having a sequence with homology to the human Neuregulin-1 protein sequence and that has at least some biological activity of the human Neuregulin-1 protein. Methods  
5 for determining whether a particular protein shares biological activity with human Nrg-1 are detailed in the examples. Preferably the homology is at least 80%, more preferably at least 85%, 90%, 95% or even more than 97%. Homology is determined as percent amino acid identity when the sequence is lined up side by side with the human sequence. In an embodiment, Neuregulin-1 is a naturally occurring homologue (genetic variation found in  
10 nature) of Neuregulin-1 such as a variant of human Neuregulin-1 or of mouse Neuregulin-1. In determining whether a polypeptide is substantially homologous to a Nrg-1 polypeptide, sequence similarity may be determined by conventional algorithms, which typically allow introduction of a small number of gaps in order to achieve the best fit. In particular, "percent homology" of two polypeptides or two nucleic acid sequences  
15 is determined using the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (J. Mol. Biol. 215:403-410, 1990). BLAST nucleotide searches may be performed with the NBLAST program to obtain nucleotide sequences homologous to a nucleic acid molecule of the invention. Equally, BLAST protein  
20 searches may be performed with the XBLAST program to obtain amino acid sequences that are homologous to a polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST is utilized as described in Altschul et al. (Nucleic Acids Res. 25:3389-3402, 1997). When utilizing BLAST and Gapped BLAST programs,

the default parameters of the respective programs (e.g., XBLAST and NBLAST) are employed. See <http://www.ncbi.nlm.nih.gov> for more details.

The term "Nrg-ICD" refers to a carboxyl terminal sequence portion of Neuregulin-1 that can be found in a cell or cell extract after the cytoplasmic portion of a Neuregulin-1 protein has been cleaved by a protease in the cell. See, for example, SEQ ID NO: 1. This protein lacks the amino terminal portion of Nrg-1, preferably lacks at least 50,000 daltons of the extracellular region, and more preferably the entire extracellular region. In an embodiment, Nrg-1 ICD includes at least 5, 10, 20 or more amino acids of the transmembrane region. Preferably the Nrg-ICD has a molecular weight of about 35,000 Daltons. The term "about" in this context means, as a minimum range, the normal variability encountered in an electrophoresis assay used for measuring protein size. Preferably the protein has an absolute molecular weight of between 25,000 daltons and 45,000 daltons. More preferably the protein has an absolute molecular weight of between 30,000 daltons and 40,000 daltons. In one embodiment, the protein is produced from Neuregulin-1 in cells such as 293T or 293S cells by one or more proteases present in these cells.

In another embodiment the Nrg-ICD has a molecular weight of about 53,000 daltons, preferably between 43,000 to 63,000 daltons and more preferably between 48,000 to 58,000 daltons. In one embodiment the protein is produced from Neuregulin-1 by proteolytic action.

The term "Eos" refers to a protein known by that name and all functional equivalents. For example, see "Eos: a novel member of the Ikaros gene family expressed predominantly in the developing nervous system" by Honma et al (FEBS Letters 447: 76-80, 1999).

The term "PDS-95" refers to a protein that is said to help build the physical scaffolding of the synapse that cells use to transmit the chemical messenger, or neurotransmitter, known as glutamate, to a target cell. The protein also apparently matures other aspects of the synapse -- enhancing the clustering of glutamate receptors on the target cells receiving the chemical messenger, increasing the number and size of the dendritic spines that hold glutamate receptors, and increasing the number of glutamate



neurotransmitters emitted from the releasing cell. This protein has been described by David S. Bredt et al (Science, November 17, 2000 edition).

IV. Other Definitions

To facilitate review of the embodiments and to provide greater clarity to the  
5 claims the following definitions for often-used terms are provided, which are consistent with usage in the field.

“Neuregulin-1 gene” (also “Neg-1 gene”) is a gene described herein, the extreme mutant forms of which are associated with neurological disease states. This definition is understood to include the various sequence polymorphisms that exist, wherein nucleotide  
10 substitutions in the gene sequence may affect the functioning of the gene product. This term relates primarily to an isolated coding sequence, but also can include some or all of the flanking regulatory elements and/or intron sequences.

“Nrg-1 protein” is a protein encoded by the human Nrg-1 cDNA. This definition is understood to include the various sequence polymorphisms that exist, wherein amino  
15 acid substitutions in the protein sequence do not completely abolish the functions of the protein.

“cDNA” refers to complementary DNA, which lacks internal, non-coding segments (introns) and lacks regulatory sequences, which determine transcription. cDNA is synthesized in the laboratory by reverse transcription from messenger RNA extracted  
20 from cells.

“DNA” refers to deoxyribonucleic acid. DNA is a long chain polymer which comprises the genetic material of most living organisms (some viruses have genes comprising ribonucleic acid). The repeating units in DNA polymers are four different nucleotides, each of which comprises one of the four bases, adenine, guanine, cytosine  
25 and thymine bound to a deoxyribose sugar to which a phosphate group is attached. Triplets of nucleotides, referred to as codons, in DNA molecules code for amino acids that are linked in a polypeptide. The term codon also is used for the corresponding (and complementary) sequences of three nucleotides in the mRNA into which the DNA sequence is transcribed. A standard nomenclature for DNA bases is employed.

“Nrg-1 Effector” refers to a molecule that has an effect on the Nrg-1 signaling pathway, and includes without limitation, Nrg-1 agonists, Nrg-1 antagonists, intracellular factors that interact with Nrg-1, intrinsic membrane proteins that interact with Nrg-1, extrinsic membrane proteins that interact with Nrg-1 and pharmaceuticals that are found to interact with any of the parts of the Neuregulin-1 signaling pathway.

“Homology” The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared times 100. For example, if 6 of 10, of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

“Hybridizations” preferably are performed under stringent conditions, for example, at a salt concentration of no more than 1M and a temperature of at least 25 degrees C. For example, a condition of 5X SSPE (750 mM NaCl, 50 mM NaPhosphate, 5 mM EDTA, pH 7.4) and a temperature of 25-30°C are suitable for allele-specific probe hybridizations.

“Hybridization probes” are oligonucleotides capable of binding in a base-specific manner to a complementary strand of nucleic acid. Such probes include peptide nucleic acids, as described in Nielsen et al., Science 254, 1497-1500 (1991).

“Isolated” means that that the material has been removed from its original environment. For example, a naturally occurring DNA molecule present in a living animal is not isolated, but the same DNA molecule, separated from some or all of the coexisting materials in the natural system, is isolated.

An “isolated nucleic acid” refers to the predominant species present. That is, on a molar basis the isolated nucleic acid it is more abundant than any other individual species in the composition. Preferably, an isolated nucleic acid comprises at least about 50, 80 or 90 percent (on a molar basis) of all macromolecular species present. Most preferably, the

object species is purified to “essential homogeneity” namely, that contaminant species cannot be detected in the composition by conventional detection methods.

“ORF” means open reading frame, which contains a series of nucleotide triplets (codons) coding for amino acids without any termination codons. These sequences are  
5 usually translatable into protein.

A “pathway gene” encodes a product, which may be regulatory RNA or a protein that is associated with the Nrg-1 gene product or with the biochemical pathways that extend to (for example, upstream signals) or from (for example, downstream signals) the Nrg-1 gene product.

10 “PCR” means polymerase chain reaction. This describes a technique in which cycles of denaturation, annealing with primer, and then extension with DNA polymerase are used to amplify the number of copies of a target DNA sequence.

“Polymorphism” refers to the occurrence of two or more genetically determined alternative sequences or alleles in a population. A polymorphic marker or site is the locus  
15 at which divergence occurs. Preferred markers have at least two alleles, each occurring at frequency of greater than 1%, and more preferably greater than 10% or 20% of a selected population. A polymorphic locus may be as small as one base pair. The first identified allelic form may be designated arbitrarily as a reference and other allelic forms designated as alternative or variant alleles. The allelic form occurring most frequently in a  
20 selected population is sometimes referred to as the wild type form. Diploid organisms may be homozygous or heterozygous for allelic forms. A diallelic polymorphism has two forms. A triallelic polymorphism has three forms.

“Primer” refers to a single-stranded oligonucleotide capable of acting as a point of initiation of template-directed DNA synthesis under appropriate conditions (i.e., in the  
25 presence of four different nucleoside triphosphates and an agent for polymerization, such as, DNA or RNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. The appropriate length of a primer depends on the intended use of the primer but typically ranges from 15 to 30 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with

the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template. The term primer site refers to the area of the target DNA to which a primer hybridizes. The term primer pair means a set of primers including a 5' upstream primer that hybridizes with the 5' end of the DNA  
5 sequence to be amplified and a 3', downstream primer that hybridizes with the complement of the 3' end of the sequence to be amplified.

The term "purified" does not necessitate absolute purity but relative purity. A reference to a protein as "purified" means that the protein is more pure with respect to the same protein in its natural environment within a cell or extracellular space from which it  
10 was purified.

A "reporter gene" encodes a sequence that produces a detectable substance such as mRNA, protein, or the activity of another reporter molecule encoded by the reporter gene. Examples of reporter gene sequences include the sequences encoding the enzymes  $\beta$ -galactosidase and luciferase.

15 A "reporter molecule", as defined herein, is a molecule or atom, which, by its chemical nature, provides an identifiable signal allowing detection. A reporter molecule may be encoded by a reporter gene. Detection can be either qualitative or quantitative. The present invention contemplates using any commonly used reporter molecules including radionucleotides, enzymes, biotin, psoralens, fluorophores, chelated heavy  
20 metals, and luciferin. The most commonly used reporter molecules are either enzymes, fluorophores, or radionucleotides linked to molecules to be detected. Commonly used enzymes include horseradish peroxidase, alkaline phosphatase, glucose oxidase and .alpha.-galactosidase, among others. Substrates for use with an enzyme may be chosen based on their ability to form a detectable colored product by the enzyme acting upon the  
25 substrate. For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for horseradish peroxidase, 1,2-phenylenediamine, 5-aminosalicylic acid or toluidine is commonly used. The methods of using hybridization probes and protein conjugates that contain such labels are well known.

A "single nucleotide polymorphism" usually arises due to substitution of one  
30 nucleotide for another at the polymorphic site. A transition is the replacement of one

purine by another purine or one pyrimidine by another pyrimidine. A transversion is the replacement of a purine by a pyrimidine or vice versa. Single nucleotide polymorphisms also can arise from a deletion of a nucleotide or an insertion of a nucleotide relative to a reference allele.

5           Additional definitions of specific terms used in receptor signaling biology are, for example, found in Mao et al., *Molecular Cell*, Vol. 7, 801-809 (2001) and common terms used in molecular biology may be found in Lewin, B. "Genes IV" published by Oxford University Press.

V.       "Binding" Type Screening Methods

10           In embodiments of the invention, test substances such as peptides and synthetic chemicals are screened for their ability to interact with one or more components of the Nrg-1 system. The screen may test for binding to Nrg-1 or Eos directly, such as to an extracellular site on the Nrg-1 protein, to an intracellular site on the Nrg-1 protein, or to Eos. In a typical screen, a test substance is incubated with a specific target of the Nrg-1  
15           system and binding is detected. The term "binding" in this context means that the association (determined by measuring an association constant) between test substance and the Nrg-1 component is greater than the association between the same test substance and other protein generally. Preferably the binding between test substance and Nrg-1 component is at least 10 times as strong and more preferably is at least 100 times as  
20           strong. Human serum albumin is preferred as a reference "other protein" because a large proportion of extracellular fluid protein is serum albumin.

          In one embodiment a useful compound for screening preferably is a natural product, peptide, or other small compound capable of mimicking the effects of a naturally occurring regulator of the Neuregulin-1 system. One preferred screening method uses  
25           upregulation of PSD-95 or other gene activator, and another uses downstream signaling pathway molecules based on the above identified reactive binding partners. The screening methods may utilize Nrg-1, the PDS-95 target gene or use other related promoters to identify Nrg-1 inducers.

A particularly desirable screening test is carried out by incubating one or more ligand binding sites (typically provided as a peptide, fusion protein, Nrg-1 protein fragment or intact Nrg-1) in an aqueous solution with one or more test substances, and then determining binding between the test substance(s) and the ligand binding site.

5 Binding assays are well known to the skilled artisan. For example, U.S. numbers 5,976,814, 5,990,128 and 5,786,155 describe methods and tools for determining ligand binding, drug screening and associated techniques that are in use, and specifically are incorporated by reference in their entireties. In one embodiment, as explained in these patent documents, binding between a receptor expressed in intact cells and a test  
10 substance is used for screening.

In a preferred embodiment of testing and drug screening, a putative drug (chemical or protein) is desired that can bind to the intracellular portion of Nrg-1 protein, can bind Eos and facilitate nuclear translocation of Nrg-ICD, or that facilitates induction of PSD-95 synthesis in response to Nrg-ICD. Other types of compounds and proteins  
15 also may be detected by screening. Still others may be screened by assays that detect other biochemical behavior such as induction of synthesis of the Nrg-1 protein. In another screening methodology embodiment an intact cell that expresses the Nrg-1 protein is used and a biochemical or morphological event such as an enzyme reaction coupled to light production is detected to indicate that a test compound facilitates the  
20 ability of Nrg-1 to upregulate the PSD-95 gene.

A ligand that demonstrates specific binding in such a binding assay described here will, according to many embodiments, modulate the transcription of PSD-95. Specific Nrg-1 effectors may be discovered this way and used as pharmaceuticals to increase synapse morphology, with concomitant effects on memory and learning, in many  
25 instances. Other ligands of course can be discovered and are useful to modulate formation of PSD-95 by affecting the Nrg-1 regulatory system in a different direction.

In an embodiment, a compound for screening may be a natural product, peptide, or other small compound capable of mimicking the effects of a naturally occurring regulator that stimulates the production of Nrg-ICD or the ability of Nrg-ICD to  
30 upregulate PSD-95. In one embodiment a DNA encoding a portion of a sequence that

emulates or that binds to a biologically effective portion of the Nrg-ICD, such as a site for proteolysis or for binding to Eos is obtained from a cDNA library prepared from tissue believed to possess the Neuregulin-1 polypeptide mRNA and that can express the DNA at a detectable level. For example, Neuregulin-1 polypeptide DNA can be conveniently  
5 obtained from a cDNA library prepared from fetal brain. The polypeptide-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis. Libraries are screened with probes (such as antibodies to the Nrg-ICD polypeptide or Eos polypeptide, or oligonucleotides of about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the  
10 selected probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Wnt polypeptide is to use PCR methodology as described in section 14 of Sambrook et al.

15       Amino acid sequence variants of polypeptide useful as Nrg-1 effectors according to this embodiment can be prepared by introducing appropriate nucleotide changes into the polypeptide DNA, or by synthesis of the desired polypeptide. Such variants represent insertions, substitutions, and/or specified deletions of, residues within or at one or both of the ends of the amino acid sequence of a naturally occurring human polypeptide.

20       Preferably, these variants represent insertions and/or substitutions within or at one or both ends of the mature sequence, and/or insertions, substitutions and/or specified deletions within or at one or both of the ends of the signal sequence of the polypeptide. Any combination of insertion, substitution, and/or specified deletion is made to arrive at the final construct, provided that the final construct possesses the desired biological activity  
25 as defined herein. The amino acid changes may include post-translational processing of the polypeptide. Such processing may involve changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, and/or altering the intracellular location of the polypeptide by inserting, deleting, or otherwise affecting the leader sequence of the polypeptide.

30       Variations in the native sequence as described above can be made using any of the techniques and guidelines for conservative and non-conservative mutations as for example set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated

(site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. The nucleic acid (e.g., cDNA or genomic DNA) encoding the Wnt polypeptide is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available for this use. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

This same general scheme may be used for discovery and modification of other polypeptides that resemble natural components of the Nrg-1 system as discovered and described herein. In many embodiments, however, pharmaceutical lead compounds are screened for their abilities to bind directly to the extracellular region of Nrg-1, intracellular region of Neuregulin-1 or to Eos. Such specific binding is particularly desirable for pharmacological intervention to promote improved synapse functioning because of decreased side effects from interacting with a specific (i.e. Nrg-1) receptor.

In one desirable embodiment a compound is selected for its effect on the Nrg-1/Eos signaling pathway by looking for preferential binding to Nrg-ICD - Eos complex over Nrg-ICD alone. This differential binding is very useful for drug screening because such binding is expected to stabilize the complex and facilitate its formation. By choosing a lead compound that binds preferably to the complex, non-specific side effects of the pharmaceutical may be minimized. The phrase "that binds preferentially to" in this context means that the binding is at least 2 times, preferably at least 5 times, more preferably at least 20 times, yet more preferably at least 100 times and even more preferably more than 500 times as strong as determined by measuring and calculating an association constant at pH 7.0 at 37 °C in aqueous solution or suspension.

## VI. Genetic Therapy

In a preferred embodiment, long term control of PSD-95 protein is achieved by transgenic expression of one or more of Neuregulin-1, Eos or of a factor that affects the interaction of these proteins to form a transcription modulator. A wide range of methods are useful for transgenic expression, including, for example, introduction of nucleic acid by a virus and introduction by vesicles. Preferably the transgenic expression occurs primarily in nerve tissue. This is best carried out by administration to nervous tissue or by selectively activating gene in nervous tissue compared with other tissue.



In another desirable embodiment an extracellular compound alters PSD-95 expression by interfering with binding between Nrg-ICD and Eos. In a preferred embodiment a polynucleic acid encoding a gene for a naturally occurring antagonist of this interaction is added in an antisense format such that an antisense strand forms and binds to messenger RNA, thus blocking translation and/or triggering nuclease destruction of the RNA. Recombination using antisense technology has previously been used to inhibit expression of specific gene products in mammalian cell lines (Kasid et al., Science 243:1354-1356 1989; Khoka et al., Science 243:947-950 1989; Izant et al., Science 229:345-352 1985) including some retroviruses; (von Ruden et al., J. Virol. 63:677-682 1989; and Chang et al., J. Virol. 61:921-924 1987). Each of these methods, and later developed methods specifically are contemplated for this embodiment of the invention.

VII. Dosage, Composition and Formulation of Pharmaceuticals for Prophylaxis and Therapy

Pharmaceutical compositions contemplated for embodiments of the invention comprise ingredients in an "effective amount" to achieve increased synaptic activity. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a measurable increase in synaptic activity.

Generally speaking, an "effective amount" of a composition is that amount which produces a statistically significant effect. When determined by effects on stimulating the formation of PSD-95 in nerve cells in vitro, it is generally desirable to produce an increase in PSD-95 of at least 10%, 25% or even more than 50%, as compared to cells grown in the absence of added effector. For therapeutic uses, an "effective amount" is the amount of the composition comprising the effector required to provide a clinically significant increase in a measurable function such as freedom from tremors, memory ability and the like. Such amounts will depend, in part, on the particular condition to be treated and other factors evident to those skilled in the art. For example in memory function, an improvement is manifested as a statistically significant difference using a

test. This can be seen as, for example, a 10-20% or more increase a test response. General guidance for treatment regimens is obtained from experiments carried out in animal models of the disease of interest.

Toxicity and therapeutic efficacy of such compounds also may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD50 and ED50. Compounds that exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p1). Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety, which are sufficient to maintain the desired effects.

In cases of local administration or selective uptake, the effective local concentration of the drug might not be related to plasma concentration. The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician. The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active compounds into preparations, which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the agent(s) of embodiments may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations, which can be used orally, include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for

oral administration should be in dosages suitable for such administration. For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner. For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents, which increase the solubility of the compounds to allow for the preparation of highly, concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use. The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides. In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by

implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A preferred pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied. Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols. Many of the compounds of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, transdermal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

5 Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into an affected area, often in a depot or sustained release formulation. Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with an antibody specific for affected cells. The liposomes will be targeted to and taken up selectively by the cells.

10 The compositions may, if desired, be presented in a pack or dispenser device, which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical  
15 carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

#### VIII. DNA-Based Diagnoses

One embodiment of the invention is the application of Nrg-1 DNA sequence  
20 information presented herein for genetic testing, carrier detection and prenatal diagnosis for genetic predisposition to weak synaptic function, hypersensitive synaptic functioning or other phenomena. Individuals carrying mutations in or near (within 5, 10, 20, 35, 50 or even more than 75 bases of) the peptide cleavage site, DNA binding site, Eos binding site or one of the nuclear binding sites of the Nrg-1 gene may be detected at the DNA level  
25 using any of a variety of techniques. For such a diagnostic procedure, a biological sample of the subject, which biological sample contains either DNA or RNA derived from the subject, is assayed for the presence of a mutant Nrg-1 gene. Suitable biological samples include samples containing genomic DNA or RNA obtained from body cells, such as those present in peripheral blood, urine, saliva, tissue biopsy, surgical specimen,  
30 amniocentesis samples and autopsy material. The detection in the biological sample of

either a mutant Nrg-1 gene or a mutant Nrg-1 RNA may be performed by a number of methodologies, as outlined below.

A preferred embodiment of such detection techniques is the polymerase chain reaction amplification of reverse transcribed RNA (RT-PCR) of RNA isolated from bone marrow cells or other cells followed by direct DNA sequence determination of the products. The presence of one or more nucleotide difference between the obtained sequence and a consensus DNA sequence, and especially, differences in the portion of the nucleotide sequence that encode the region that binds to Eos, are taken as indicative of a potential Nrg-1 gene mutation.

The detection of specific DNA mutations may be achieved by methods such as hybridization using specific oligonucleotides (Wallace et al. (1986). Cold Spring Harbor Symp. Quant. Biol. 51:257-261), direct DNA sequencing (Church and Gilbert (1988). Proc. Natl. Acad. Sci. USA 81:1991-1995), the use of restriction enzymes (Flavell et al. (1978). Cell 15:25), discrimination on the basis of electrophoretic mobility in gels with denaturing reagent (Myers and Maniatis (1986). Cold Spring Harbor Symp. Quant. Biol. 51:275-284), RNase protection (Myers et al. (1985). Science 230:1242), chemical cleavage (Cotton et al. (1985). Proc. Natl. Acad. Sci. USA 85:4397-4401), and the ligase-mediated detection procedure (Landegren et al., 1988).

Oligonucleotides specific to normal or mutant sequences are chemically synthesized using commercially available machines, labeled radioactively with isotopes (such as <sup>32</sup>P) or non-radioactively (with tags such as biotin (Ward and Langer et al. (1981). Proc. Natl. Acad. Sci. USA 78:6633-6657), and hybridized to individual DNA samples immobilized on membranes or other solid supports by dot-blot or transfer from gels after electrophoresis. The presence or absence of these specific sequences are determined optically by methods such as autoradiography or fluorometric (Landegren, et al., 1989) or colorimetric reactions (Gebeyehu et al. (1987). Nucleic Acids Res. 15:4513-4534).

Sequence differences between normal and mutant functional sites of the gene also may be revealed by the direct DNA sequencing method of Church and Gilbert (1988).

Cloned DNA segments may be used as probes to detect specific DNA segments. The

sensitivity of this method is greatly enhanced when combined with PCR (Wrichnik et al., 1987; Wong et al., 1987; Stoflet et al. (1988). Science 239:491-494). In this approach, a sequencing primer which lies within the amplified sequence is used with double-stranded PCR product or single-stranded template generated by a modified PCR. The sequence  
5 determination is performed by conventional procedures with radiolabeled nucleotides or by automatic sequencing procedures with fluorescent tags.

Sequence alterations occasionally may generate fortuitous restriction enzyme recognition sites or may eliminate existing restriction sites. Changes in restriction sites are revealed by the use of appropriate enzyme digestion followed by conventional gel-  
10 blot hybridization (Southern (1975). J. Mol. Biol. 98:503). DNA fragments carrying the site (either normal or mutant) are detected by their reduction in size or increase of corresponding restriction fragment numbers. Genomic DNA samples may also be amplified by PCR prior to treatment with the appropriate restriction enzyme; fragments of different sizes are then visualized under UV light in the presence of ethidium bromide  
15 after gel electrophoresis.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing reagent. Small sequence deletions and insertions can be visualized by high-resolution gel electrophoresis. For example, a PCR product with small deletions is  
20 clearly distinguishable from a normal sequence on an 8% non-denaturing polyacrylamide gel (WO 91/10734, Nagamine et al. Am. J. Hum. Genet. 45:337-339 (1989). DNA fragments of different sequence compositions may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific "partial-melting" temperatures  
25 (Myers et al. (1985). Science 230:1242). Alternatively, a method of detecting a mutation comprising a single base substitution or other small change could be based on differential primer length in a PCR. For example, an invariant primer could be used in addition to a primer specific for a mutation. The PCR products of the normal and mutant genes can then be differentially detected in acrylamide gels.



In addition to conventional gel-electrophoresis and blot-hybridization methods, DNA fragments may also be visualized by methods where the individual DNA samples are not immobilized on membranes. The probe and target sequences may be both in solution, or the probe sequence may be immobilized (Saiki et al. Proc. Nat. Acad. Sci. USA 86:6230-6234 (1989)). A variety of detection methods, such as autoradiography involving radioisotopes, direct detection of radioactive decay (in the presence or absence of scintillant), spectrophotometry involving calorogenic reactions and fluorometry involved fluorogenic reactions, may be used to identify specific individual genotypes.

Other more recent methods to detect genetic polymorphism are useful for embodiments of the invention. A particularly good example is that disclosed in U.S. No. 5,856,104 issued to Chen et al. January 5, 1999, the contents of which specifically are incorporated by reference in its entirety.

If more than one mutation is frequently encountered in the Nrg-1 gene, a system capable of detecting such multiple mutations would be desirable. For example, a PCR with multiple, specific oligonucleotide primers and hybridization probes may be used to identify all possible mutations at the same time (Chamberlain et al. (1988). Nucl. Acids Res. 16:1141-1155 (1988)). The procedure may involve immobilized sequence-specific oligonucleotides probes (Saiki et al. Proc. Nat. Acad. Sci. USA 86:6230-6234) (1989).

## IX. DNA Vectors Useful for Improved Synaptic Function

Other embodiments of the invention provide recombinant DNA vectors comprising the desirable DNA sequences that affect synapse function and other functions.

With the provision of the Nrg-1 cDNA and/or Eos cDNAs and the understanding of the role and mechanisms of the coded proteins in the Neuregulin-1 control system, vectors that contain Nrg-1 DNA may be used for diagnosis and therapy. Furthermore, the DNA sequence of the Nrg-1 cDNA and polymorphic or mutated Nrg-1 cDNAs isolated from patients can be manipulated in studies to further understand the expression of the gene and the function of its product. In this way, further ligands useful for embodiments

of the invention and mechanisms involved in control of synapse plasticity and related biological functions can be discovered.

Mutant versions of Nrg-1 DNA and Eos DNA at specific sites of proteolytic cleavage, nuclear transport signals, Eos binding, Nrg-1 binding, and DNA binding, and  
5 other nucleic acid sequences may be isolated based upon information contained herein.

This information may be used to detect alteration in expression patterns in terms of relative quantities, tissue specificity and functional properties of the encoded Nrg-1 protein in individuals to determine their PSD-95 induction status. Partial or full-length DNA sequences, which encode Nrg-1 may be ligated into bacterial expression vectors.

10 Methods for expressing large amounts of protein from a cloned gene that is introduced into *Escherichia coli* (*E. coli*) may be utilized to purify, localize and study the function of proteins involved in the regulation. For example, fusion proteins consisting of amino terminal peptides encoded by a portion of the *E. coli* *lacZ* or *trpE* gene linked to Nrg-1 proteins may be used to prepare polyclonal and monoclonal antibodies against these  
15 proteins. Thereafter, these antibodies may be used to purify proteins by immunoaffinity chromatography, in diagnostic assays to quantitate the levels of protein and to localize proteins in tissues and individual cells by immunofluorescence.

Intact native protein also may be produced in *E. coli* in large amounts for functional studies. Native proteins can be produced in bacteria by placing a strong,  
20 regulated promoter and an efficient ribosome binding site upstream of the cloned gene. If low levels of protein are expressed, additional steps may be taken to increase protein production; if high levels of protein are expressed, purification is easier. Suitable methods are presented in Sambrook et al. (1989). In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y. and are well known in the art. Often, proteins  
25 expressed at high levels are found in insoluble inclusion bodies. Methods for extracting proteins from these aggregates are described by Sambrook et al. (1989). In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y. Vector systems suitable for the expression of *lacZ* fusion genes include the pUR series of vectors (Ruther and Muller-Hill (1983). *EMBO J.* 2:1791), pEX1-3 (Stanley and Luzio (1984). *EMBO J.* 3:1429) and  
30 pMR100 (Gray et al. (1982). *Proc. Natl. Acad. Sci. USA* 79:6598). Vectors suitable for the production of intact native proteins include pKC30 (Shimatake and Rosenberg (1981).

Nature (London) 292:128), pKK177-3 (Amann and Brosius (1985). Gene 40:183.) and pET-3 (Studiar and Moffatt (1986). J. Mol. Biol. 189:113).

Methods and plasmid vectors for producing fusion proteins and intact native proteins in bacteria are described in Sambrook et al. (1989). In Molecular Cloning: A  
5 Laboratory Manual, Cold Spring Harbor, N.Y. (ch. 17, herein incorporated by reference). Such fusion proteins may be made in large amounts, are easy to purify, and can be used to elicit antibody response. Nrg-1 fusion proteins may be isolated from protein gels, lyophilized, ground into a powder and used as an antigen. The DNA sequence can also be transferred from its existing context in pREP4 to other cloning vehicles, such as other  
10 plasmids, bacteriophages, cosmids, animal viruses and yeast artificial chromosomes (YACs) (Burke et al. (1987). Science 236:806-812). These vectors may then be introduced into a variety of hosts including somatic cells, and simple or complex organisms, such as bacteria, fungi (Timberlake and Marshall (1989). Science 244:1313-1317), invertebrates, plants (Gasser and Fraley (1989). Science 244:1293), and pigs  
15 (Pursel et al. (1989). Science 244:1281-1288), which cell or organisms are rendered transgenic by the introduction of the heterologous Nrg-1 or Eos cDNA, or portions thereof.

For expression in mammalian cells, the cDNA sequence may be ligated to heterologous promoters, such as the simian virus (SV)40, promoter in the pSV2 vector  
20 (Mulligan and Berg, 1981), and introduced into cells, such as monkey COS-1 cells (Gluzman (1981). Cell 23:175-182), to achieve transient or long-term expression. The stable integration of the chimeric gene construct may be maintained in mammalian cells by biochemical selection, such as neomycin (Southern and Berg (1982). J. Mol. Appl. Genet. 1:327-341) and mycophenolic acid (Mulligan and Berg, 1981).

25 DNA sequences can be manipulated with standard procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences, site-directed sequence-alteration via single-stranded bacteriophage intermediate or with the use of specific oligonucleotides in combination with PCR.

The cDNA sequence (or portions derived from it) or a mini gene (a cDNA with an intron and its own promoter) is introduced into eukaryotic expression vectors by conventional techniques. These vectors are designed to permit the transcription of the cDNA eukaryotic cells by providing regulatory sequences that initiate and enhance the transcription of the cDNA and ensure its proper splicing and polyadenylation. Vectors containing the promoter and enhancer regions of the SV40 or long terminal repeat (LTR) of the Rous Sarcoma virus and polyadenylation and splicing signal from SV40 are readily available (Mulligan et al., 1981; Gorman et al. (1982). *Proc. Natl. Acad. Sci. USA* 78:6777-6781). The level of expression of the cDNA can be manipulated with these types of vector, either by using promoters that have different activities (for example, the baculovirus pAC373 can express cDNAs at high levels in *S. frugiperda* cells (Summers and Smith (1985). In *Genetically Altered Viruses and the Environment*, Fields et al. (Eds.) 22:319-328, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y) or by using vectors that contain promoters amenable to modulation, for example, the glucocorticoid-responsive promoter from the mouse mammary tumor virus (Lee et al. (1982). *Nature* 294:228). The expression of the cDNA can be monitored in the recipient cells 24 to 72 hours after introduction (transient expression).

In addition, some vectors contain selectable markers such as the gpt (Mulligan and Berg, 1981) or neo (Southern and Berg (1982). *J. Mol. Appl. Genet.* 1:327-341) bacterial genes. These selectable markers permit selection of transfected cells that exhibit stable, long-term expression of the vectors (and therefore the cDNA). The vectors can be maintained in the cells as episomal, freely replicating entities by using regulatory elements of viruses such as papilloma (Schafner (1980). *Proc. Natl. Acad. Sci. USA* 77:2163-2167). *Proc. Natl. Acad. Sci. USA* 77:2163-2167). *Mol. Cell Biol.* 1:486) or Epstein-Barr (Sugden et al. (1985). *Mol. Cell Biol.* 5:410). Such episomal vectors are exemplified by the pREP4 Epstein-Barr virus vector in which the cDNA library described in Example 2 herein was constructed. Alternatively, one can also produce cell lines that have integrated the vector into genomic DNA. Both of these types of cell lines produce the gene product on a continuous basis. One can also produce cell lines that have amplified the number of copies of the vector (and therefore of the cDNA as well) to create cell lines that can produce high levels of the gene product (Alt et al. (1978). *J. Biol. Chem.* 253:1357).

The transfer of DNA into eukaryotic, in particular human or other mammalian cells, is now a conventional technique. The vectors are introduced into the recipient cells as pure DNA (transfection) by, for example, precipitation with calcium phosphate (Graham and vander Eb (1973). *Virology* 52:466) or strontium phosphate (Brash et al. (1987). *Mol. Cell Biol.* 7:2013), electroporation (Neumann et al. (1982). *EMBO J* 1:841), lipofection (Felgner et al. (1987). *Proc. Natl. Acad. Sci. USA* 84:7413), DEAE dextran (McCuthan et al. (1968). *J. Natl. Cancer Inst.* 41:351), microinjection (Mueller et al. (1978). *Cell* 15:579), protoplast fusion (Schafner (1980). *Proc. Natl. Acad. Sci. USA* 77:2163-2167), or pellet guns (Klein et al., 1987). Alternatively, the cDNA can be introduced by infection with virus vectors. Systems are developed that use, for example, retroviruses (Bernstein et al. (1985). *Gen. Engr'g* 7:235), adenoviruses (Ahmad et al. (1986). *J. Virol.* 57:267.), or Herpes virus (Spaete et al. (1982). *Cell* 30:295).

These eukaryotic expression systems can be used for studies of the Nrg-1 and Eos genes and mutant forms of these genes, the Nrg-1 and Eos proteins and mutant forms of these proteins. Such uses include, for example, the identification of regulatory elements located in the 5' region of the Nrg-1 gene on genomic clones that can be isolated from human genomic DNA libraries using the information contained in the present invention. The eukaryotic expression systems may also be used to study the function of the normal complete protein, specific portions of the protein, or of naturally occurring or artificially produced mutant proteins. Naturally occurring mutant proteins exist in patients with Nrg-1, while artificially produced mutant proteins can be designed by site directed mutagenesis as described above. These latter studies may probe the function of any desired amino acid residue in the protein by mutating the nucleotide coding for that amino acid.

Using the above techniques, the expression vectors containing the Nrg-1 gene sequence or fragments or variants or mutants thereof can be introduced into human cells, mammalian cells from other species or non-mammalian cells as desired. The choice of cell is determined by the purpose of the treatment. Generally, transfer into cells that forms synapses is preferred.

The following is provided as one exemplary method to express Nrg-1 polypeptide from the cloned Nrg-1 cDNA sequences in mammalian cells. Cloning vector pXTI, commercially available from Stratagene, contains the Long Terminal Repeats (LTRs) and a portion of the GAG gene from Moloney Murine Leukemia Virus. The position of the viral LTRs allows highly efficient, stable transfection of the region within the LTRs. The vector also contains the Herpes Simplex Thymidine Kinase promoter (TK), active in embryonal cells and in a wide variety of tissues in mice, and a selectable neomycin gene conferring G418 resistance. Two unique restriction sites BglII and XhoI are directly downstream from the TK promoter. Nrg-1 cDNA, including the entire open reading frame for the Nrg-1 protein and the 3' untranslated region of the cDNA is cloned into one of the two unique restriction sites downstream from the promoter.

The ligated product is transfected into mouse NIH 3T3 cells using Lipofectin (Life Technologies, Inc.) under conditions outlined in the product specification. Positive transfectants are selected after growing the transfected cells in 600  $\mu$ g/ml G418 (Sigma, St. Louis, Mo.). The protein is released into the supernatant and may be purified by standard immunoaffinity chromatography techniques using antibodies raised against the Nrg-1 protein, as described below.

Expression of the Nrg-1 protein in eukaryotic cells may also be used as a source of proteins to raise antibodies. The Nrg-1 protein may be extracted following release of the protein into the supernatant as described above, or, the cDNA sequence may be incorporated into a eukaryotic expression vector and expressed as a chimeric protein with, for example, beta-globin. Antibody to  $\beta$ -globin is thereafter used to purify the chimeric protein. Corresponding protease cleavage sites engineered between the  $\beta$ -globin gene and the cDNA are then used to separate the two polypeptide fragments from one another after translation. One useful expression vector for generating  $\beta$ -globin chimeric proteins is pSG5 (Stratagene). This vector encodes rabbit  $\beta$ -globin.

The recombinant cloning vector, according to this invention, then comprises the selected DNA of the DNA sequences of this invention for expression in a suitable host. The DNA is operatively linked in the vector to an expression control sequence in the recombinant DNA molecule so that the Nrg-1 polypeptide can be expressed. The

expression control sequence may be selected from the group consisting of sequences that control the expression of genes of prokaryotic or eukaryotic cells and their viruses and combinations thereof. The expression control sequence may be specifically selected from the group consisting of the lac system, the trp system, the tac system, the trc system,  
5 major operator and promoter regions of phage lambda, the control region of fd coat protein, the early and late promoters of SV40, promoters derived from polyoma, adenovirus, retrovirus, baculovirus and simian virus, the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, the promoter of the yeast alpha-mating factors and combinations thereof.

10 Retroviruses have been considered the preferred vector for experiments in gene therapy, with a high efficiency of infection and stable integration and expression (Orkin et al. (1988). Prog. Med. Genet. 7:130). The full length Nrg-1 gene, optionally with the Eos gene, or DNA can be cloned into a retroviral vector and driven from either its endogenous promoter or from the retroviral LTR (long terminal repeat). Other viral transfection  
15 systems may also be utilized for this type of approach, including Adeno-Associated virus (AAV) (McLaughlin et al. (1988). J. Virol. 62:1963), Vaccinia virus (Moss et al. (1987). Annu. Rev. Immunol. 5:305), Bovine Papilloma virus (Rasmussen et al. (1987). Methods Enzymol. 139:642) or members of the herpesvirus group such as Epstein-Barr virus (Margolske et al. (1988). Mol. Cell. Biol. 8:2837-2847).

20 The mouse is an extremely useful experimental organism for experiments with transgenic technology. The cloning of the mouse Nrg-1 homologue permits generation of a mouse model for Nrg-1 by targeted gene replacement in mouse embryonic stem cells (Sedivy and Joyner (1992). In Gene Targeting, W. H. Freeman and Company, New York). This in turn, will facilitate the study of the abnormal developmental processes  
25 leading to the pleiotropic phenotype of synaptic processes.

The host cell, which may be transfected with the vector of this invention, may be selected from the group consisting of E. coli, Pseudomonas, Bacillus subtilis, Bacillus stearothermophilus or other bacilli; other bacteria; yeast; fungi; insect; mouse or other animal; or plant hosts; or human tissue cells.

It is appreciated that for mutant or variant DNA sequences, similar systems are employed to express and produce the mutant product.

*Diagnosis of PSD-95 protein based Synapse Function using the Nrg-1 Gene Sequence*

One embodiment of the invention is a screening method to determine if a subject  
5 carries a Nrg-1 gene with a mutation in a functional region and or to determine resultant  
altered synapse strength and activity. The method comprises the steps of: providing a  
biological sample obtained from the subject, which sample includes DNA or RNA,  
providing an assay for detecting in the biological sample the presence of at least one  
member from the group consisting of a mutant Nrg-1 gene and a mutant Nrg-1 RNA. A  
10 preferred embodiment of this method is described wherein the assay comprises a method  
selected from the group consisting of: hybridization with oligonucleotides; PCR  
amplification of the Nrg-1 gene or a part thereof using oligonucleotide primers; RT-PCR  
amplification of the Nrg-1 RNA or a part thereof using oligonucleotide primers, and  
direct sequencing of the Nrg-1 gene of the subject's genome using oligonucleotide  
15 primers. When the availability of intron sequence data from the splice sites of the human  
Nrg-1 gene and polymerase chain reactions for the amplification of these sequences from  
genomic DNA, as provided by this invention, will permit the analysis of these regions for  
potential splice site mutations. Furthermore, the efficiency of these molecular genetic  
methods should permit a more rapid classification of patients than is possible with the  
20 labor intensive method of classical complementation analysis.

A embodiment is a method for screening a subject to assay for the presence of a  
mutation in a functional site of a Nrg-1 gene, comprising the steps of: providing a  
biological sample of the subject which sample contains cellular proteins and providing an  
immunoassay for determining synapse stimulation quality of Nrg-1 protein in the  
25 biological sample, based on differential binding to antibodies. Most preferred is an  
embodiment that used monoclonal antibodies that react with ligand binding site(s) on the  
extracellular portion of the Nrg-1 protein. The generation of monoclonal antibodies using  
peptide, chimeric protein, or entire intact Nrg-1 protein uses routine methodology.

In further embodiments, a subject is screened to determine his or her  
30 polymorphism for the Nrg-1 gene. This information is used to predict propensity to



alterations in PDS-95 and to synapse activity. Various techniques for assaying genetic polymorphism are known, as exemplified in U.S. Nos. 6,074,831; 5,364,759; 5,614,364; and 5,856,104, the contents of which specifically are incorporated by reference in their entireties, particularly with respect to their descriptions on how to detect polymorphism of a gene.

Another embodiment of the invention is an immunoassay for detecting predisposition induction of the PSD-95 gene, comprising antibodies that specifically bind Nrg-ICD protein, wherein the antibodies are selected from the group consisting of monoclonal antibodies and polyclonal antibodies. Techniques for using the antibodies in a kit (that is, in a package with an instructional chart or package insert) are routine and are contemplated as further embodiments of the invention.

Through the manipulation of the nucleotide sequence of the human or murine cDNAs provided by this invention by standard molecular biology techniques, variants of the proteins described herein may be made which differ in precise amino acid sequence from the disclosed proteins yet which maintain the essential characteristics of the proteins or which are selected to differ in some characteristics from these proteins. Such variants are another aspect of the present invention.

#### *Nucleic Acids Useful for Embodiments of the Invention*

A large variety of isolated human and animal Nrg-1 and Eos cDNA sequences, and partial sequences that hybridize with these genes are useful for detecting an individual's tendency for PDS-95 regulation and synapse activity according to embodiments of the invention. Representative nucleic acid sequences in this context have been shown in the figures. Still further variants of these sequences and sequences of nucleic acids that hybridize with those shown in the figures also are comprehended for use in detection and alteration of PDS-95 status/tendency, as are the genomic genes from which these cDNAs are derived.

Many of the contemplated variant DNA molecules include those created by standard DNA mutagenesis techniques, for example, M13 primer mutagenesis. Details of these techniques are provided in Sambrook et al. (1989). In Molecular Cloning: A

Laboratory Manual, Cold Spring Harbor, N.Y. By the use of such techniques, variants may be created which differ in minor ways from those disclosed. DNA molecules and nucleotide sequences which are derivatives of those specifically disclosed herein and which differ from those disclosed by the deletion, addition or substitution of nucleotides while still encoding a protein which possesses the functional characteristic of the Nrg-1 protein are comprehended by this invention. Also within the scope of this invention are small DNA molecules, which are derived from the disclosed DNA molecules. Such small DNA molecules include oligonucleotides suitable for use as hybridization probes or polymerase chain reaction (PCR) primers. As such, these small DNA molecules will comprise at least a segment of an Nrg-1 cDNA molecule or the Nrg-1 gene and, for the purposes of PCR, will comprise at least a 10-15 nucleotide sequence and, more preferably, a 15-30 nucleotide sequence of the Nrg-1 cDNA or the Nrg-1 gene. DNA molecules and nucleotide sequences which are derived from the disclosed DNA molecules as described above may also be defined as DNA sequences which hybridize under stringent conditions to the DNA sequences disclosed, or fragments thereof.

Hybridization conditions corresponding to particular degrees of stringency vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing DNA used. Generally, the temperature of hybridization and the ionic strength (especially the sodium ion concentration) of the hybridization buffer will determine the stringency of hybridization. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook et al. (1989). In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y., chapters 9 and 11, herein incorporated by reference. By way of illustration only, a hybridization experiment may be performed by hybridization of a DNA molecule (for example, a deviation of the Nrg-1 cDNA) to a target DNA molecule (for example, the Nrg-1 cDNA) which has been electrophoresed in an agarose gel and transferred to a nitrocellulose membrane by Southern blotting (Southern (1975). *J. Mol. Biol.* 98:503), a technique well known in the art and described in Sambrook et al. (1989). In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y. Hybridization with a target probe labeled with isotopic P (32) labelled-dCTP generally is carried out in a solution of high ionic strength such as 6x SSC at a temperature that is 20 –25 °C, below the melting temperature,  $T_m$ , described below. For such Southern hybridization experiments where

the target DNA molecule on the Southern blot contains 10 ng of DNA or more, hybridization is typically carried out for 6-8 hours using 1-2 ng/ml radiolabeled probe (of specific activity equal to  $10^9$  CPM/mug or greater). Following hybridization, the nitrocellulose filter is washed to remove background hybridization. The washing conditions should be as stringent as possible to remove background hybridization but to retain a specific hybridization signal. The term " $T_m$ " represents the temperature above which, under the prevailing ionic conditions, the radiolabeled probe molecule will not hybridize to its target DNA molecule. The  $T_m$  of such a hybrid molecule may be estimated from the following equation (Bolton and McCarthy (1962). Proc. Natl. Acad. Sci. USA 48:1390):  $T_m = 81.5^\circ\text{C} - 16.6(\log_{10} \text{ of sodium ion concentration}) + 0.41(\%G+C) - 0.63(\% \text{ formamide}) - (600/l)$  where  $l$ =the length of the hybrid in base pairs. This equation is valid for concentrations of sodium ion in the range of 0.01M to 0.4M, and it is less accurate for calculations of  $T_m$  in solutions of higher sodium ion concentration. The equation also is valid for DNA having G+C contents within 30% to 75%, and also applies to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Ch. 11 of Sambrook et al. (1989). In Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y.

Thus, by way of example of a 150 base pair DNA probe derived from a section of 150 base pairs of the open reading frame of the Nrg-1 cDNA (with an assumed % GC=45%), a calculation of hybridization conditions required to give particular stringencies may be made as follows:

For this example, it is assumed that the filter will be washed in 0.3 X SSC solution following hybridization, thereby sodium ion =0.045M; % GC=45%; Formamide concentration=0  $l$ =150 base pairs (EQU1) and so  $T_m$  =74.4 degrees C.

The  $T_m$  of double-stranded DNA decreases by 1-1.5  $^\circ\text{C}$  with every 1% decrease in homology (Bonner et al. (1973). J. Mol. Biol. 81:123). Therefore, for this given example, washing the filter in 0.3x SSC at 59.4-64.4  $^\circ\text{C}$  will produce a stringency of hybridization equivalent to 90%; that is, DNA molecules with more than 10% sequence variation relative to the target Nrg-1 cDNA will not hybridize. Alternatively, washing the hybridized filter in 0.3x SSC at a temperature of 65.4-68.4  $^\circ\text{C}$  will yield a hybridization

stringency of 94%; that is, DNA molecules with more than 6% sequence variation relative to the target Nrg-1 cDNA molecule will not hybridize. The above example is given entirely by way of theoretical illustration. One skilled in the art will appreciate that other hybridization techniques may be utilized and that variations in experimental conditions will necessitate alternative calculations for stringency.

In preferred embodiments of the present invention, stringent conditions may be defined as those under which DNA molecules with more than 25% sequence variation (also termed "mismatch") will not hybridize. In a more preferred embodiment, stringent conditions are those under which DNA molecules with more than 15% mismatch will not hybridize, and more preferably still, stringent conditions are those under which DNA sequences with more than 10% mismatch will not hybridize. In a most preferred embodiment, stringent conditions are those under which DNA sequences with more than 6% mismatch will not hybridize.

The degeneracy of the genetic code further widens the scope of the present invention as it enables major variations in the nucleotide sequence of a DNA molecule while maintaining the amino acid sequence of the encoded protein. The nucleotide sequence of the Nrg-1 cDNA could be changed at various positions to other codons without affecting the amine acid composition of the encoded protein or the characteristics of the protein. The genetic code and variations in nucleotide codons for particular amine acids is presented in Tables 4 and 5. Based upon the degeneracy of the genetic code, variant DNA molecules may be derived from the cDNA molecules disclosed herein using standard DNA mutagenesis techniques as described above, or by synthesis of DNA sequences. DNA sequences, which do not hybridize under stringent conditions to the cDNA sequences disclosed by virtue of sequence variation based on the degeneracy of the genetic code, are included in an embodiment.

Table 3

The Genetic Code				
	First	Second	Third	
5	Position	Position	Position	
	(5') T	C	A G (3')	
	T	Phe	Ser Tyr	Cys T
		Phe	Ser Tyr	Cys C
10		Leu	Ser Stop	Stop A (och)
		Leu	Ser Stop (amb)	Trp G
	C	Leu	Pro His	Arg T
		Leu	Pro His	Arg C
		Leu	Pro Gln	Arg A
15		Leu	Pro Gln	Arg G
	A	Ile	Thr Asn	Ser T
		Ile	Thr Asn	Ser C
		Ile	Thr Lys	Arg A
		Met	Thr Lys	Arg G
20	G	Val	Ala Asp	Gly T
		Val	Ala Asp	Gly C
		Val	Ala Glu	Gly A
		Val (Met)	Ala Glu	Gly G

25 “Stop (och)” stands for the ochre termination triplet, and “Stop (amb)” for the amber. ATG is the most common initiator codon; GTG usually codes for valine, but it can also code for methionine to initiate an mRNA chain.

Table 4

The Degeneracy of the Genetic Code		
5	Number of	Total
	Synonymous	Number of
	Codons	Codons
	Amino Acid	
	6 Leu, Ser, Arg	18
	4 Gly, Pro, Ala, Val, Thr	20
10	3 Ile	3
	2 Phe, Tyr, Cys, His, Gln, Glu, Asn, Asp, Lys	18
	1 Met, Trp	2
	Total number of codons for amino acids	
15	61	
	Number of codons for termination	
	3	
	Total number of codons in genetic code	
20	64	

One skilled in the art will recognize that the DNA mutagenesis techniques described above may be used not only to produce variant DNA molecules, but will also facilitate the production of proteins which differ in certain structural aspects from the Nrg-1 and Eos protein, yet which proteins are clearly derivative of this protein and which maintain the essential characteristics of the Nrg-1 and Eos proteins. Newly derived proteins may also be selected in order to obtain variations on the characteristic of the Nrg-1 and Eos proteins, as will be more fully described below. Such derivatives include those with variations in amino acid sequence including minor deletions, additions and substitutions.

While the site for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the

performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed protein variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence as described above are well  
5 known.

Amino acid substitutions are typically of single residues; insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e., a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any  
10 combination thereof may be combined to arrive at a final construct. Obviously, the mutations that are made in the DNA encoding the protein must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure.

Substitutional variants are those in which at least one residue in the amino acid  
15 sequence has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 5 when it is desired to finely modulate the characteristics of the protein. Table 5 shows amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions.

20                      Table 5

		Original Residue	Conservative Substitutions
		Ala	ser
25		Arg	lys
		Asn	gln, his
		Asp	glu
		Cys	ser
		Gln	asn
30		Glu	asp

	Gly	pro
	His	asn; gln
	Ile	leu, val
	Leu	ile; val
5	Lys	arg; gln; glu
	Met	leu; ile
	Phe	met; leu; tyr
	Ser	thr
	Thr	ser
10	Trp	tyr
	Tyr	trp; phe
	Val	ile; leu

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Substantial changes in function or immunological identity are made by selecting  
15 substitutions that are less conservative than those in Table 5, i.e., selecting residues that  
differ more significantly in their effect on maintaining (a) the structure of the polypeptide  
backbone in the area of the substitution, for example, as a sheet or helical conformation,  
(b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the  
side chain. The substitutions which in general are expected to produce the greatest  
20 changes in protein properties will be those in which (a) a hydrophilic residue, e.g., seryl  
or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl,  
phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other  
residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histadyl,  
is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a  
25 residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not  
having a side chain, e.g., glycine.

The effects of these amino acid substitutions or deletions or additions may be  
assessed for derivatives of the Nrg-1 protein by analyzing the ability of the derivative  
proteins to complement the sensitivity to DNA cross-linking agents exhibited by Nrg-1  
30 cells. These assays may be performed by transfecting DNA molecules encoding the  
derivative proteins into Nrg-1 cells as described above.



The Nrg-1 gene, Eos gene, Nrg-1 cDNA, Eos cDNA, DNA molecules derived therefrom and the protein encoded by the cDNA and derivative DNA molecules may be utilized in aspects of both the study of Nrg-1 (and Eos) and for diagnostic and therapeutic applications related to these proteins. Utilities of the present invention include, but are not limited to, those utilities described in the examples presented herein. Those skilled in the art will recognize that the utilities herein described are not limited to the specific experimental modes and materials presented and will appreciate the wider potential utility of this invention.

The detection of specific DNA mutations may be achieved by methods such as hybridization using specific oligonucleotides (Wallace et al. (1986). Cold Spring Harbor Symp. Quant. Biol. 51:257-261), direct DNA sequencing (Church and Gilbert (1988). Proc. Natl. Acad. Sci. USA 81:1991-1995), the use of restriction enzymes (Flavell et al. (1978). Cell 15:25), discrimination on the basis of electrophoretic mobility in gels with denaturing reagent (Myers and Maniatis (1986). Cold Spring Harbor Symp. Quant. Biol. 51:275-284), RNase protection (Myers et al. (1985). Science 230:1242), chemical cleavage (Cotton et al. (1985). Proc. Natl. Acad. Sci. USA 85:4397-4401), and the ligase-mediated detection procedure (Landegren et al., 1988).

Oligonucleotides specific to normal or mutant sequences are chemically synthesized using commercially available machines, labeled radioactively with isotopes (such as  $^{32}\text{P}$ ) or non-radioactively (with tags such as biotin (Ward and Langer et al. Proc. Natl. Acad. Sci. USA 78:6633-6657 1981), and hybridized to individual DNA samples immobilized on membranes or other solid supports by dot-blot or transfer from gels after electrophoresis. The presence or absence of these specific sequences are visualized by methods such as autoradiography or fluorometric or colorimetric reactions (Gebeyehu et al. Nucleic Acids Res. 15:4513-4534 1987).

Sequence differences between normal and mutant forms of gene regions that correlate to functional sites as described herein, also may be revealed by the direct DNA sequencing method of Church and Gilbert (1988). Cloned DNA segments may be used as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR (Stoflet et al. Science 239:491-494, 1988). In this

approach, a sequencing primer which lies within the amplified sequence is used with double-stranded PCR product or single-stranded template generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotides or by automatic sequencing procedures with fluorescent tags.

5           Sequence alterations may occasionally generate fortuitous restriction enzyme recognition sites or may eliminate existing restriction sites. Changes in restriction sites are revealed by the use of appropriate enzyme digestion followed by conventional gel-blot hybridization (Southern (1975). J. Mol. Biol. 98:503). DNA fragments carrying the site (either normal or mutant) are detected by their reduction in size or increase of  
10       corresponding restriction fragment numbers. Genomic DNA samples may also be amplified by PCR prior to treatment with the appropriate restriction enzyme; fragments of different sizes are then visualized under UV light in the presence of ethidium bromide after gel electrophoresis.

          Genetic testing based on DNA sequence differences may be achieved by detection  
15       of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing reagent. Small sequence deletions and insertions can be visualized by high-resolution gel electrophoresis. For example, a PCR product with small deletions is clearly distinguishable from a normal sequence on an 8% non-denaturing polyacrylamide gel (WO 91/10734, Nagamine et al. Am. J. Hum. Genet. 45:337-339 1989). DNA fragments  
20       of different sequence compositions may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific "partial-melting" temperatures (Myers et al. Science 230:1242 1985). Alternatively, a method of detecting a mutation comprising a single base substitution or other small change could be based on differential primer length  
25       in a PCR. For example, an invariant primer could be used in addition to a primer specific for a mutation. The PCR products of the normal and mutant genes can then be differentially detected in acrylamide gels.

          In addition to conventional gel-electrophoresis and blot-hybridization methods, DNA fragments may also be visualized by methods where the individual DNA samples  
30       are not immobilized on membranes. The probe and target sequences may be both in

solution, or the probe sequence may be immobilized (Saiki et al., Proc. Nat. Acad. Sci. USA 86:6230-6234 1989). A variety of detection methods, such as autoradiography involving radioisotopes, direct detection of radioactive decay (in the presence or absence of scintillant), spectrophotometry involving calorigenic reactions and fluorometry involved fluorogenic reactions, may be used to identify specific individual genotypes.

If more than one mutation is frequently encountered in the Nrg-1 gene, a system capable of detecting such multiple mutations would be desirable. For example, a PCR with multiple, specific oligonucleotide primers and hybridization probes may be used to identify all possible mutations at the same time (Chamberlain et al. Nucl. Acids Res. 16:1141-1155 (1988)). The procedure may involve immobilized sequence-specific oligonucleotides probes (Saiki et al., Proc. Nat. Acad. Sci. USA 86:6230-6234 1989).

### **Examples**

The following materials and methods were used for examples 1-7.

Materials and Methods:

#### **Broadband sound stimulation.**

Mice were subjected to broadband sound stimulation in a foam-lined soundproof room (Industrial Acoustics) as described previously (Ohlemiller et al., *Hear. Res.* 149: 239-247 (2000)). The sound exposure apparatus consisted of a 21 × 21 × 11 cm wire cage mounted on a pedestal inserted into a B&K 3921 turntable. A Motorola KSN1020A piezo ceramic speaker (four total) was attached to each side of the frame. Sound was generated by two General Radio 1310 generators and band-passed at 4.0–45 kHz by Krohn-Hite 3550 filters. The overall sound level was measured at the center of the cage using a B&K 2231 sound level meter set to broadband (0.2 Hz–70 kHz). All procedures were approved by the Central Institute for the Deaf Animal Studies Committee.

#### **Cellular fractionation and western blotting.**

Nuclear fractions were prepared using Nuclear and Cytoplasmic Extraction Reagents (Pierce). Next, 40 µg of nuclear protein was separated by 12% SDS-PAGE, transferred to nitrocellulose membranes (Schleicher & Schuell) and probed with antibodies to Nrg-ICD (sc-348, Santa Cruz Biotechnology), histone H1 (sc-8616, Santa Cruz Biotechnology) and eIF5 (sc-282, Santa Cruz Biotechnology). For the western blotting of PSD-95, homer and SV2 proteins, 120 µg total protein from cell lysates of spiral ganglia was separated by

10% SDS-PAGE, transferred to nitrocellulose membrane and probed with a mouse monoclonal antibody to PSD-95 (BD Transduction Laboratories) and later with either a rabbit polyclonal antibody to SV2A (Synaptic Systems) or a rat anti-homer polyclonal antibody (Chemicon). To quantify bands on western blots, an image of the blot was  
5 imported into Adobe Photoshop and a background density was determined for each lane using the histogram program. This value was subtracted from the density of the 52-kDa, 35-kDa or PSD-95 band. A statistical analysis between two groups was determined using a paired Student's *t*-test.

**Plasmid constructs.**

10 pcDNA3.1 expressing full-length Nrg-1, Nrg-1c, ICDc, ICDA, Eos-C and pGL3 plasmids containing various PSD-95 promoters (pGL3a, pGL3b, pGL3c, pGL3d and pGL3e) were prepared by PCR. The primer pair for fusing full-length Nrg-1 to the HA epitope was: 5': ACCAT GTCTG AGGGA GCTGG CGGGA GGT (SEQ ID NO: 16); 3': TCATA CAGCG TAGTC TGGGA CGTCG TATGG GTA (SEQ ID NO: 17). The same 5'-primer  
15 was used for Nrg-1c. The 3'-primer for Nrg-1c was: 3': TCACC TTTCA CTATG AGGAG AGTCT CTGTA GGA (SEQ ID NO: 18). The primer pair for expressing ICDc only was:

5': ACCAT GAAAA CCAAG AAACA GCGGC A (SEQ ID NO: 19); 3': ACCTT TCACT ATGAG GAGAG TCTCT (SEQ ID NO: 20). The primer pair for expressing  
20 ICDA only was: 5': CACCA TGTAT GTATC AGCCA TGACC ACC (SEQ ID NO: 21); 3': TACAG CAATA GGGTC TTGGT TAGC (SEQ ID NO: 22). The primer pair for Eos-C was: 5': TCGCG ATCCC AGATG AGTCT CAGCA CTGAA GCC (SEQ ID NO: 23); 3': CT CGAGC TAGCC CACCT TATGC TCCCC CCG (SEQ ID NO: 24).

The exact sequence data for human PSD-95 promoter was obtained from GenBank  
25 ([AF156495](#)). Various deletions were made and cloned into *Bgl*II and *Hind*III sites of pGL3 (Promega). The primer pairs for various 5' deletions were as follows. Three PSD-95 promoter sense primers: 5' (start at -2493): ATTAG ATCTA GG TAG GAGGG TGACT TGCT (SEQ ID NO: 25); 5' (start at -1614): TAAAG ATCTA GCTGG GAAAA GAGAC GGTTA GCA (SEQ ID NO: 26); 5' (start at -1575): ATTAG ATCTT CCGCT  
30 GTGTC AAGGG AGAAC AGGCG (SEQ ID NO: 27). One PSD-95 promoter antisense primer: 5': GTTAA GCTTA CTAAA TTCCA GCTGT GAGTA AAGT (SEQ ID NO: 28). Primers for PCR-based site-directed mutagenesis (mutated sequences are

underlined):

5' (first site): GTAAGGACTTGAAAACCAGGGTGAG (SEQ ID NO: 29); 5' (second site): CGTTCACCTCCTGGAAAAGGCACAGGATCC (SEQ ID NO: 30); 5' (third site): CCGGACGG GTATGAAAACACCTGATC (SEQ ID NO: 31); 5' (fourth and fifth sites):  
5 CAGAGGGGTGAAA TCTGACTTGTCCAGAGCCAGCT GAAAAAAGAGACG (SEQ ID NO: 32); 3': CTGAGGCTCGG GGGCGGAAG (SEQ ID NO: 33). All construct sequences were confirmed by DNA sequencing.

#### **Cell cultures and immunostaining.**

All culture media and supplements were purchased from Invitrogen. 293T cells, PS1<sup>-</sup>  
10 PS2<sup>-</sup> cells and SGNs were cultured, transfected and immunostained as described (Cohen-Cory S. et al., *Science* 290:750-54 (2002)). The following antibodies were used: anti-Nrg-ICD (sc-348, 1:2,000, Santa Cruz Biotechnology); anti-HA-tag (262K, 1:1,000, Cell Signaling Technology); anti-Flag-tag (F-3165, 1:1,000, Sigma); anti-PSD-95 (610496, 1:1,000, BD Transduction Laboratories, or 05-494, 1:2,000, Upstate). The  
15 staining was viewed with a multiphoton microscope (BioLab).

#### **Gene array.**

Total RNA isolated from untreated and 50-mM KCl-treated SGN cultures was labeled with <sup>32</sup>P using the Atlas Pure Total RNA Labeling System (Clontech) and hybridized to Atlas Mouse 1.2 Arrays (Clontech). After a high-stringency wash and autoradiography,  
20 differences between the two hybridization patterns were quantified and listed in the tables (Tables 1 and 2).

#### **Electrophoretic mobility gel shift assay (EMSA).**

Ten micrograms of a nuclear extract were incubated for 20 min at room temperature with 10<sup>5</sup>–10<sup>6</sup> cpm of <sup>32</sup>P-labeled oligonucleotide probes corresponding to the consensus  
25 GATA-1, GATA-2/3, Ik1/2, MZF-1 or Sp1 binding sequence in binding buffer consisting of 10 mM Tris-HCl (pH 7.5), 15 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol and 1 mg/ml poly(dI/dC). The reaction mixture was separated on a 4% nondenatured polyacrylamide gel and detected by autoradiography. Competition assays were carried out using a 100-fold excess of unlabeled corresponding oligonucleotide.  
30 Super-gel-shift assays were carried out by adding 2 µl of the corresponding antibody to the binding buffer, then incubating the reaction for 1 h at 4 °C. Sequences for two additional oligonucleotide probes used in generating the data shown in Figures 3 and 4

are Ik1/2 (CAGGGAATCTCCCTCTCCAT) (SEQ ID NO: 34) and a mutated Ikaros site (CAGGTAATCTCCCTCTCCAT) (SEQ ID NO: 35).

**Luciferase assays.**

293T cells were plated onto 12-well plates the day before transfection. E16 cortical  
5 neurons were plated onto 24-well plates 2 d before transfection. The combination of 2 µg of pGL3 luciferase plasmids, 0.1 µg *Renilla* luciferase reporter vector (to normalize for transfection efficiency, Promega) and 2 µg of expression plasmids for Nrg-1, Nrg-1c, ICDa, ICDc, Eos or Pegasus were transfected using LipofectAMINE (Invitrogen). To depolarize neurons, some transfected groups were treated with 50 mM KCl 24 h after  
10 transfection. After 48 h, cells were lysed and luciferase activity measured by the dual luciferase assay system (Promega).

**Immunoprecipitation and SDS-PAGE.**

Immunoprecipitation was carried out using a kit (Pierce) according to the manufacturer's instructions. Immuno-precipitated material was released from the beads by boiling each  
15 sample for 5 min in loading sample buffer (50 mM TrisCl, 100 mM dithiothretol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Apparent molecular mass was estimated by comparing the relative mobility of immunoreactive bands to those of prestained SDS-PAGE standards (Bio-Rad).

**ChIP assay.**

20 ChIP analysis was performed following a protocol provided by Upstate Biotechnology. For 293T cells, 24 h after transfection, cells from two 10-cm dishes were fixed with 1% formaldehyde. For SGNs, six spiral ganglia were fixed 1, 30 and 60 min after the sound stimulation. After 5-min fixation, cells were washed extensively with ice-cold PBS and lysed for 10 min in lysis buffer (Upstate Biotechnolgy). Chromatin was sheared by  
25 sonication to an average size of approximately 1 kilobase and pre-cleared for 2 h at 4 °C with salmon sperm DNA-saturated protein G Sepharose. Chromatin solution was precipitated overnight at 4 °C using 20 µl anti-Nrg-ICD, Flag or calretinin (Zymed Laboratories Inc.) or beads alone. Immune complexes were collected with salmon sperm DNA-saturated protein G Sepharose for 1 h and washed extensively following the  
30 manufacturer's protocol. Input and immunoprecipitated chromatin were incubated at 65 °C overnight to reverse crosslinks. After proteinase K digestion, DNA was extracted with phenol/chloroform and precipitated with ethanol. Precipitated DNAs was analyzed by

PCR (35 cycles). For amplify mouse PSD-95 promoter, we used two promoter-specific primers:

MP95C5 (-TGTCCAGAGCCAAGCTGGGAA-) (SEQ ID NO: 36) and MP95C3 (-CACTATACAG AGACAGTCCAT) (SEQ ID NO:37); to amplify the human the PSD-95 promoter, HP95C5 (-AGGACTTGGAACCAAGGGTGAGGCCCA-) (SEQ ID NO: 38) and HP95C3 (-CCCTGAACTT GCTAACCGTCTCTTTTCC-) (SEQ ID NO: 39).

***Example 1: Increase of PSD-95 and nuclear Nrg-ICD by sound stimuli.***

DNA microarray analysis was used to identify candidate down-stream genes regulated by nuclear Nrg-ICD in response to neuronal depolarization in cultured spiral ganglion neurons (SGNs). Of 6,000 genes on the array, 32 were differentially expressed between control (untreated) SGNs and SGNs depolarized by exposure to 50 mM KCl: 6 were upregulated and 26 were downregulated (Table 1 and 2). PSD-95 mRNA was the most strongly upregulated mRNA in depolarized SGN samples (to 17.8-fold higher than control levels). This response was examined *in vivo*, by testing whether PSD-95 was upregulated in SGNs after sound stimulation in C57BL/6J mice. Six hours after sound stimulation, a marked increase in PSD-95 immunoreactivity was observed in the SGN postsynaptic terminals beneath the layer of inner hair cells (IHC), and a moderate increase was observed beneath the outer hair cells (OHC) (Fig. 8a). The upregulation of PSD-95 protein was confirmed by western blot analysis of SGNs under both moderate (86 dB sound pressure level (SPL) for 2 min) and strong (110 dB SPL for 5 min) stimulation (Fig. 8b). Quantitative results from three independent experiments using the strong stimulation are shown (Fig. 8c). Levels of two other synaptic proteins, homer and synaptic vesicle protein-2 (SV2), were not affected by the auditory stimulation (Fig. 8b), indicating that not all synaptic proteins are affected by this type of synaptic activation.

Examination of SGN total cell extracts by western blot analysis with an antibody to Nrg-ICD (anti-Nrg-ICD) revealed two fragments: (i) a full-length ICD that includes ICDc and ICDa (52kDa) and (ii) ICDa alone (35kDa), which is the end fragment of the 'a' form of Nrg-ICD (Fig. 8d,e). Thirty minutes after the moderate sound stimulation, there were no changes in either of these two Nrg-ICD fragments (Fig. 8e). The specificity of this anti-Nrg-ICD was tested in a previous study (Cohen-Cory S. et al., *Science* 290:750-54 (2002)). However, when we examined the SGN nuclear fraction, we observed

an increase in both fragments 30 min after the sound stimulation (Fig. 8f,g). The purity of the nuclear and cytoplasmic fractions was demonstrated with antibodies specific for histone H1, a nuclear protein, and for elongation initiation factor-5 (eIF5), a cytoplasmic protein.

## 5    **Example 2: Association of Nrg-ICD and Eos with PSD-95 promoter**

Although Nrg-ICD lacks a DNA binding motif, it shows strong transcriptional activity and can bind to proteins with zinc-finger domains or a similar motif, such as an LIM domain (Bao, J et al., *Soc. Neurosci. Abstr.* 23, 562.1, (1997); Bao, J et al., *J. Biol. Chem.* 161:1133-41 (2003); Wang et al., *J. Biol. Chem.* 273: 20525-34 (1998)). The PSD-  
10    95 promoter contains binding sites for the zinc-finger transcription factors GATA-1, GATA-2/3, Ik1/2, MZF-1 and Sp-1 (Stathakis et al., *J. Neurochem.* 73:2250-65 (1999)). To determine whether Nrg-ICD binds to the PSD-95 promoter through one of these factors, EMSAs were performed on nuclear extracts purified from 293T cells (a non-neuronal human kidney cell line) expressing Nrg-1 (tagged with a hemagglutinin (HA)-  
15    derived epitope at its C terminus). Of these five isotope-labeled oligonucleotide probes, only the Ik1/2 probe was supershifted by antibodies to Nrg-ICD or HA; these antibodies had no effect on the mobility of the other four complexes (for example, Fig. 9a for SP-1; Fig. 9b). Thus, Nrg-ICD is present in the complex binding to the Ik1/2 probe.

The Ik1/2 site is a putative regulation site for transcriptional factors in the Ikaros  
20    family of hematopoietic-specific zinc finger proteins (Georgopoulos K. et al., *Nat. Rev. Immunol.* 2:162-74 (2002)). Two recently identified members of this family, Eos and Pegasus, show broad tissue distribution and are expressed in the nervous system (Honma Y. et al., *FEBS Lett.* 447:76-80 (1999); Perdomo et al., *J. Biol. Chem.* 275:38347-54 (2000)). To determine whether Nrg-ICD regulates transcriptional activity of the PSD-95  
25    promoter through either of these factors, Nrg-1 was tested in combination with Eos or Pegasus to determine if it would affect the expression of a luciferase reporter gene placed under the control of the PSD-95 promoter in 293T cells (Buonanno A et al., *Curr. Opin. Neurobiol.* 11:287-96 (2001); Carraway and Burden, *Curr. Opin. Neurobiol.* 5:606-12 (1995); Bao et al., *J. Biol. Chem.* 161:1133-41 (2003)). Studies have indicated that the  
30    expression of Nrg-1 in transfected 293T cells results in the formation of cleavage products (Nrg-1 extracellular domain and Nrg-ICDs) in the absence of stimuli. Under



these conditions, coexpression of Eos and Nrg-1 with the reporter resulted in approximately fourfold higher PSD-95 promoter activity. There was no significant effect on PSD-95 promoter activity with Eos alone, Pegasus alone or Pegasus in combination with Nrg-1. Nrg-1 alone increased PSD-95 promoter activity by approximately twofold (Fig. 9c), probably as a result of endogenous Eos in 293T cells (Perdomo et al., *J. Biol. Chem.* 275:38347-54 (2000)). There are five Ikaros sites within the PSD-95 promoter (Fig. 9d). A series of progressive 5' deletion promoter-reporter constructs were made to identify regions responsible for upregulation of PSD-95 promoter activity by Nrg-1 and Eos. These included constructs with all five Ikaros sites (pGL3a), a single Ikaros site (pGL3b) or no Ikaros sites (pGL3c). No significant differences in PSD-95 promoter activity were found between the pGL3a and pGL3b constructs, whereas deletion of the last Ikaros site (39 nucleotides) in the pGL3c reduced promoter activity markedly, to the basal level. To be certain that only the Ikaros site was responsible for upregulation of PSD-95 promoter activity by Nrg-1 and Eos in neurons, all five Ikaros sites were mutated (from GGGAA (SEQ ID NO: 40) to GAAAA (SEQ ID NO: 41)) by PCR-based site-directed mutagenesis. A pronounced decrease in PSD-95 promoter activity was observed in neurons only when all five Ikaros sites were mutated (Fig. 9e). These data indicate that Nrg-1 and Eos upregulate PSD-95 promoter activity by binding to Ikaros sites.

**Example 3: Interaction between Nrg-ICD and Eos**

Nrg-1 and Eos are expressed in the same neurons, such as SGNs, dorsal root ganglion neurons and hippocampus neurons (Bao et al., *J. Biol. Chem.* 161:1133-41 (2003); Honma Y. et al., *FEBS Lett.* 447:76-80 (1999)). To confirm that Nrg-ICD and Eos form a complex binding to the Ikaros site, coimmunoprecipitation assays were carried out on 293T cells coexpressing the two proteins. Eos was coimmunoprecipitated by an antibody to Nrg-ICD (Fig. 10a, left) and both Nrg-ICD (52 kDa) and Nrg-ICDa (35 kDa) were coimmunoprecipitated by an anti-Flag antibody that recognizes the Flag tag on Eos (Fig. 10a, right). EMSA experiments provided more direct evidence for the association of the Nrg-ICD-EOS protein complex to the Ikaros binding motif. Nuclear extracts from 293T cells cotransfected with Nrg-1 and Eos were prepared and then analyzed by EMSA. The Ikaros probe was supershifted upon incubation with anti-Nrg-ICD or the anti-Flag (Fig. 10b, lanes 4 and 6) and, notably, was 'super-supershifted' upon addition of

antibodies to both Nrg-ICD and Flag (Fig. 10b, lane 5). As expected, Nrg-ICD and Eos did not bind to the mutated Ikaros site (Fig 3b, lane 3).

**Example 4: Nrg-ICD–EOS signaling is independent of  $\gamma$ -secretase**

Two vectors were constructed that expressed only specific isoforms: Nrg-ICD, ICDc and ICDA (Fig. 10c). Experiments were designed to compare the effects of Nrg-1, ICDc and ICDA on the expression of a luciferase reporter gene placed under the control of the PSD-95 promoter in 293T cells (open columns in Fig. 10d). Expression of ICDA alone with this reporter increased PSD-95 promoter activity by approximately 3.3-fold, whereas ICDc alone did not have a significant effect. Coexpression of Eos with Nrg-1, ICDc and ICDA increased PSD-95 promoter activity by 3.3-, 3.0- and 4.0-fold respectively (Fig. 10d). Thus, ICDA is the most potent activator for upregulating PSD-95 promoter activity, and ICDc can activate the PSD-95 promoter only after overexpression of Eos.

The experiment was repeated in a cell line lacking both (PS1<sup>-</sup> PS2<sup>-</sup>; filled columns in Fig. 10d). Although the effects of Nrg-1, ICDc and ICDA on PSD-95 promoter activity were less pronounced in this cell line, the full-length Nrg-1 still activated the PSD-95 promoter (1.6-fold alone and 2.3-fold with Eos). These data indicated that the cleavage of Nrg-1 from the membrane is likely to have a role in this nuclear signaling pathway, but not an essential one. The cleavage of the full-length Nrg-1 was studied in stable cell lines expressing wild-type (PSwt) or loss-of-function mutant forms of PS1 (dAsp). The full-length Nrg-1, ICD (52 kDa) and ICDA (35 kDa) were found in PSwt cells after Nrg-1 transfection. As with the cleavage of another membrane protein, ErbB4 in dAsp cells (Lee et al., *J. Biol. Chem.* 277:6318-23 (2002)), a band approximately the size of ICD accumulated markedly owing to the failure of intramembrane cleavage (ICDm). Notably, ICDA was still present in the dAsp cells, indicating that intramembrane cleavage of full-length ICD from Nrg-1 probably depends on the presence of PS1, but the cleavage of ICDA from Nrg-1 does not. A similar observation was made with 293T cells expressing Nrg-1 after treatments with the specific  $\gamma$ -secretase inhibitors, ComE and L686 (Fig. 10e), confirming that two different cleavage pathways exist for ICD and ICDA.

**Example 5: Induction of PSD-95 by Nrg-ICD–EOS signaling**

To determine whether the Nrg-ICD–Eos nuclear signaling pathway alone is sufficient to regulate the expression of endogenous PSD-95 *in vivo*, we carried out chromatin immunoprecipitation (ChIP) assays with antibodies to Nrg-ICD, or to the Flag tag for Eos

detection, using 293T cells. No recruitment of Eos to the PSD-95 promoter was observed in cells transfected with the Eos expression vector alone; weak recruitment of Nrg-1 in cells expressing Nrg-1 alone; but strong recruitment of Nrg-ICD and Eos in cells expressing both Nrg-1 and Eos (Fig. 11a). The expression levels of Nrg-1 and Eos in these transfected cells were similar (Fig. 11b). This result was confirmed by western blotting. PSD-95 was detected in cells expressing Nrg-1 and Eos or Nrg-1 alone, but not in cells expressing Eos or Pegasus alone (Fig. 11c). PSD-95 induction in transfected cells was directly visualized by immunocytochemistry (Fig. 11d). Notably, in the transfected cells expressing Eos alone, only particle-like immunostaining for Eos was observed, whereas more diffuse and 'membrane-associated' patterns were observed in cells cotransfected with Eos and Nrg-1, which closely resembled the cellular distribution of Nrg-ICD. Similar results were obtained in transfected PS1<sup>-</sup> PS2<sup>-</sup> cells (Fig. 11e), which supports the idea that PSD-95 induction by Nrg-ICD nuclear signaling is independent of  $\gamma$ -secretase.

In neurons cultured for 5 d, PSD-95 was not detected by immunocytochemistry. However, in neurons cotransfected with Nrg-1 and Eos, 100% (103 of 103 neurons) showed strong PSD-95 immunoreactivity, whereas in neurons transfected with Nrg-1 alone, only 24% (6 of 25 neurons) showed PSD-95 immunoreactivity, perhaps because only a small percentage of neurons expressed endogenous Eos. In neurons transfected with Eos alone, 15% (26 of 172 neurons) showed PSD-95 immunoreactivity (Fig. 11f). As in transfected 293T cells, the cellular localization of Eos shifted from nuclear 'dots' to a membrane-associated localization when Nrg-1 was present. The change in the subcellular localization of Eos was not due to high levels of Nrg-1 in cotransfected neurons. In neurons transfected with the Eos mammalian expression vector alone, membrane-associated Eos localization was occasionally observed. In each case, weak Nrg-ICD immunostaining was seen, indicating that Nrg-1 was endogenously expressed in these neurons at a level that was sufficient to alter the cellular localization of Eos (Fig. 11g). Thus, Nrg-ICD nuclear signaling alone is sufficient to induce endogenous PSD-95 expression, and changes in the cellular localization of Eos are mediated by the physical interaction of Eos with Nrg-ICD.

***Example 6: Activation of PSD-95 promoter by Nrg-ICD-EOS***

SGN nuclear extracts were isolated from mice at 1 or 30 min after sound stimulation and

EMSA was used to examine binding to the Ik1/2 probe. Binding was consistently enhanced in SGN nuclear extracts 30 min after stimulation (Fig. 12a). The complete elimination of the Ik1/2 band by competing nonradioactive Ik1/2 oligonucleotide, and the supershift of the band by an antibody to Nrg-ICD, confirmed the identity of the Ik1/2 complex on the gel. This finding was verified by ChIP assays. Nrg-ICD was recruited to the PSD-95 promoter 30 min after sound stimulation in SGN nuclear extracts. The recruitment was transient, disappearing 1 h after stimulation (Fig. 12b). The recruitment of Nrg-ICD to the PSD-95 promoter in SGNs was specific because calretinin, a protein abundant in SGNs, was not recruited to the PSD-95 promoter. To further confirm that the Nrg-ICD–EOS signaling pathway is critical for activity-dependent regulation of PSD-95 in neurons, an experiment was designed to test whether perturbing interactions between Nrg-ICD and Eos decreased PSD-95 promoter activity in neurons. First, the 'a' isoform was deleted from Nrg-1, producing Nrg-1c (an ICD with the 'c' isoforms in its cytoplasmic tail). Lower PSD-95 promoter activity was observed in neurons transfected with Nrg-1c and Eos as compared to neurons transfected with Nrg-1 and Eos ( $116.1 \pm 9.9$  versus  $223.6 \pm 39.4$ ). This data confirmed the findings that ICDA is the key regulator, although ICDC can regulate PSD-95 promoter activity. Second, Eos contains an N-terminal domain, which is responsible for DNA binding, and a C-terminal domain, which is responsible for protein-protein interactions (binding to Nrg-ICD). A mammalian expression vector was made to express only the C terminus of Eos (Eos-C) to block the interaction between Nrg-1 and Eos. This resulted in a marked decrease of PSD-95 promoter activity (Fig. 12c). When neurons were activated by 50 mM KCl, this rescued the ability of Nrg-1c to upregulate PSD-95 promoter activity, possibly owing to the increase of Nrg-1 intramembrane cleavage, whereas this activation did not relieve the dominant negative blockage of Eos-C. All these data are strongly suggestive of activity-dependent transcription regulation of PSD-95 by Nrg-ICD and Eos.

***Example 7:***

To gain more insight into the dynamics of regulated nuclear targeting of the Nrg-1-ICD, a series of chimeric CRD-Nrg-1s were expressed in HEK 293T cells (Fig. 6). Subcellular targeting of Nrg-1 was followed in living cells transfected with a CRD-Nrg-1 $\beta$ a-GFP fusion protein by continuous monitoring of the distribution of GFP by collecting images through the z-axis of cells (Fig. 13A). In control cells, the strongest CRD-Nrg-1-GFP

signal was detected around the cell periphery and in a single intracellular region, consistent with previous reports of Nrg-1 localization in the plasma membrane, Golgi structure, and endoplasmic reticulum (Burgess et al., J. Biol. Chem. 270:19188-96 (1995)). This pattern remained essentially unchanged for up to 2 h of continuous observation. In contrast, within 2–4 min after treatment with soluble erbB2 + erbB4, the distribution of green fluorescence changed and distinct fluorescent aggregates were seen both in peripheral regions of the cells and near Golgi-like structures. By 16 min after erbB2:erbB4 treatment, these GFP aggregates moved along discrete paths and entered the nucleus (Fig. 13, arrows).

The induced targeting of Nrg-1-ICD to the nucleus indicated that the Nrg-1-ICD might contain an identifiable NLS. Inspection of the primary sequence of Nrg-1-ICDs identified two potential NLSs. The first, NLS-1, includes the first eight amino acids after the transmembrane domain (KTKKQRKK) (SEQ ID NO: 3) and is found in all Nrg-1-ICDs. We expressed Nrg-1-ICD-GFP fusion proteins that contained or lacked these eight amino acids in 293T cells (Fig. 13B; these fusion proteins included just the ICD of Nrg-1 fused to GFP and lacked the transmembrane domain). Strong nuclear and diffuse cytoplasmic staining was seen when Nrg-1 $\beta$ c-ICD-GFP was expressed. Nrg-1 $\beta$ c-ICD $\Delta$ NLS1-GFP, lacking the eight-amino acid NLS, was distributed diffusely throughout the cells and did not concentrate in nuclei, which is consistent with a requirement for this domain for accumulating Nrg-1-ICD in nuclei. The presence or absence of the second putative NLS (PRLREKK) (SEQ ID NO: 4) had no effect on the cellular localization of GFP fusion proteins (unpublished data). To test that the erbB2:erbB4-induced nuclear targeting of Nrg-1 a-ICD was associated with proteolysis of the full-length transmembrane form of Nrg-1, we separated cytoplasmic and membrane fractions from nuclear extracts of HEK293T cells expressing a CRD-Nrg-1 $\beta$ a-HA fusion protein (full-length CRD-Nrg-1 $\beta$ a tagged at the COOH terminus with an 11-amino acid HA epitope). The Nrg-1 COOH terminus was detected by probing immunoblots with an anti-HA antibody. In cells incubated under control conditions (untreated or treated with soluble erbB2; Fig. 13C, erbB2), the ~110-kD full-length protein and several higher molecular mass bands were detected. These higher molecular mass bands likely correspond to highly glycosylated or possibly aggregated forms of Nrg-1 (Wang et al., 2001). Treatment of transfected cells

with soluble erbB2 + erbB4 resulted in increased amounts of a mostly nuclear ~50-kD protein corresponding to the Nrg-1-ICD (Fig. 13C, erbB2:B4).

**Table 1 Up-regulated genes after neuronal activity.**

Gene Name	GenBank Accession Number	Fold change
YB1 DNA Binding Protein	X57621	2.4
HHEX	Z21524	2.1
PSD-95	V61751	17.8
P180	M60778	6.4
MECP2	AF072251	3.2
Col9A2	Z22923	2.2

**Table 2 Down-regulated genes after neuronal activity.**

Gene Name	GenBank Accession Number	Fold change	
			5
CACCC Box binding protein	O36340	-5.7	
SIX2	X80338	-7.0	
SIX 1	X80339	-4.8	
MYOG	D90156	-9.9	
P38-2G4	U43918	-2.3	
PTMA	X56135	-2.1	
HMG-14	X53476	-2.1	10
SCP-1	L12721	-2.4	
NDK B	X68193	-3.9	
IBP2	X81580	-16.2	
Apo-E	M12414	-2.2	
CDH2	M31131	-3.4	
TTF1	X83974	-3.2	
SEMAPHORIN G	X97818	-6.4	
IGF-11	M14951	-2.6	15
IGFBP4	X81582	-3.9	
PROENKEPHALIN A PRECURSOR	M55181	-3.0	
FGFR1	M28998	-3.7	
MPIN	AF02185	-2.9	
ErbB2	L47239	-3.6	
G25K	O37720	-6.7	
G-PROTEIN s	Y00703	-2.2	20
NLK	M14220	-7.7	
THROMBOMODULIN	X14432	-4.4	
TGF 2	X57413	-3.2	
MYL6	Q60605	-5.3	



*PATENTS*  
*Attorney Docket No.: 104916*  
*Express Mail No. EV 535075419 US*

Every document cited is specifically incorporated in its entirety by reference.

I claim

1. A drug discovery method for identifying a compound that modulates the induction of PSD-95 by the Nrg-1/Eos signaling pathway comprising :

(i) contacting one or more test compounds with Nrg-ICD or a portion thereof, wherein the Nrg-ICD or portion thereof is encoded by a nucleic acid that hybridizes to a nucleic acid having SEQ ID NO: 1 in 5.times.SSC at 42.degree. C.; and (ii) identifying the binding between the one or more test compounds and Nrg-ICD or a portion thereof.

2. The method of claim 1, further comprising the step of exposing a cell to an identified compound from step (ii) and determining whether the identified compound modulates translocation of Nrg-ICD into the nucleus of the cell.

3. The method of claim 2, wherein modulation of translocation is measured fluorometrically.

4. The method of claim 1, further comprising the step of exposing a cell to an identified compound from step (ii) and determining whether the identified compound binds to Eos.

5. A drug discovery method for identifying a compound that modulates binding of Nrg-ICD with a binding site of Eos, comprising:

(i) contacting one or more test compounds with Nrg-ICD or a portion thereof and with at least one binding site of Eos, wherein the Nrg-ICD is encoded by a nucleic acid that hybridizes to a nucleic acid having SEQ ID NO: 1 in 5.times.SSC at 42.degree. C.;

(ii) contacting Nrg-ICD or a portion thereof with the at least one binding site of Eos in the absence of the one or more test compounds; and

(iii) identifying a difference in binding between Nrg-ICD or a portion thereof and with the at least one binding site of Eos between the contacting of (i) and the contacting of (ii).

6. The method of claim 5, wherein step (i) occurs after step (ii) by adding the one or more test compounds to a solution prepared in step (ii).

7. A drug discovery method for identifying a compound that modulates translocation of Nrg-ICD into a cell nucleus, comprising:

(i) contacting a cell with one or more test compounds; and

(ii) detecting movement of Nrg-ICD from the cell cytoplasm into the cell nucleus.

8. The method of claim 7, wherein movement of Nrg-ICD is indirectly detected by measuring the amount of Nrg-ICD in the cell nucleus after step (i).

9. The method of claim 7, wherein detection is carried out fluorometrically.
10. The method of claim 7, wherein the Nrg-ICD is produced transgenically within the cell.
11. The method of claim 9, wherein the Nrg-ICD comprises a conjugate of a polypeptide encoded by a sequence that is at least 90% homologous with SEQ ID NO: 1 and a detectable label.
12. The method of claim 9, wherein the Nrg-ICD comprises a conjugate of a polypeptide encoded by a sequence that is at least 95% homologous with SEQ ID NO: 1 and a detectable label.
13. A method for identifying a compound which promotes or inhibits translocation of Nrg-ICD across the nuclear membrane of a cell, comprising:
  - (i) transgenically expressing in cells, a polypeptide complex comprising a nuclear localization sequence of Nrg-ICD and a detectable label, wherein the localization sequence of Nrg-ICD is at least 90% homogeneous with a portion that exceeds 20 amino acids of SEQ ID NO: 1; and
  - (ii) contacting the cells with test compounds and determining whether a test compound affects translocation of Nrg-ICD across the nuclear membrane of the cell.
14. The method of claim 13, wherein the nuclear localization sequence is selected from the group consisting of SEQ ID NO: 3 [KTKKQRKK] and SEQ ID NO: 4 [PRLREKK].
15. The method of claim 13, wherein the cells are neurons.
16. The method of claim 13, wherein the detectable label is selected from the group consisting of green fluorescent protein, a chemilumiphore, an antigenic peptide sequence and a regulatory marker.
17. A method for identifying a compound that promotes or inhibits translocation of Nrg-ICD across the nuclear membrane of a cell, comprising:
  - (i) transgenically expressing in cells, a polypeptide complex comprising a nuclear localization sequence of Nrg-ICD and a regulatory marker, wherein the localization sequence of Nrg-ICD is at least 90% homogeneous with a portion that exceeds 20 amino acids of SEQ ID NO: 2 and the regulatory marker influences the expression of a gene when present within the nucleus of the cell; and

(ii) contacting the cells with test compounds and determining whether a test compound affects translocation of Nrg-ICD across the nuclear membrane of the cell.

18. The method of claim 17, wherein the regulatory marker is selected from the group consisting of a promoter and an enhancer.

19. The method of claim 17, wherein the cell nucleus comprises a foreign gene that produces a protein that conveys a selectable trait to the cell and the regulatory marker is a promoter or enhancer of that foreign gene.

20. A method for identifying a compound that modulates the proteolysis of Neu-1 to form Nrg-ICD, comprising:

- (i) incubating a cellular membrane form of Neu-1 in the presence of the compound; and
- (ii) detecting the formation of a carboxylic end portion of Neu-1 that is less than 60 kilodaltons in size.

21. The method of claim 20, wherein the cellular membrane form of Neu-1 is intact cells.

22. The method of claim 20, wherein the carboxylic end portion of Neu-1 is approximately 35 kilodaltons in size.

23. The method of claim 20, wherein detection of the carboxylic end portion comprises detection of an immunologically reactive water soluble polypeptide.

24. A method for identifying a compound that modulates gene activity by binding to an Ikaous 1/2 sequence, comprising:

- (i) providing transgenic cells that contain a reporter gene operably coupled to a promoter that comprises Ikaous 1/2 sequence;
- (ii) contacting the cells with one or more test substances; and
- (iii) detecting the induction of the reporter gene in response to one or more test substances.

25. The method of claim 24, wherein the cells transgenically express Neu-1.

26. A fusion polypeptide of a pharmaceutically active compound discovered by the method of any of claims 1 through 22, comprising a first polypeptide portion of between 8 and 50 amino acids long that exhibits binding to Nrg-ICD or Eos and a second polypeptide portion comprising a transporter moiety of between 10 and 20 amino acids long.

27. The fusion compound of claim 26, wherein the second polypeptide portion has a sequence that is selected from the group consisting of SEQ ID NO:4 [YGRKKRRQRRR] and SEQ ID NO: 5 [RQIKIWFQNRRMKWKK].

28. The fusion polypeptide of claim 26, wherein the pharmaceutically active compound binds Neu-1

29. A method for enhancing learning in an animal, comprising providing to the animal a compound that modulates the formation or translocation of Nrg-ICD into the nucleus of a nerve cell, wherein the compound is a fusion compound as described in claim 26.

30. A method for preventing neuronal excitotoxicity in an animal, comprising providing to the animal a pharmaceutical that attenuates the nuclear signaling pathway of Neu-1

31. A transgenic animal with enhanced learning capability, produced by the process of stably incorporating an exogenous Neu-1 gene into the animal and expressing the gene .

32. The transgenic animal of claim 31, wherein the added gene is expressed constitutively in nerve cells.

33. An isolated protein complex, comprising primarily of Ng-ICD and Eos.

34. A vector that comprises a gene encoding Nrg-ICD and a gene encoding Eos.

**Abstract**

Methods are provided for discovery of new lead pharmaceuticals useful for addressing diseases and other problems of hearing. Compounds that modulate PSD-95 induction are discovered via use of the Nrg-1/Eos signaling pathway. Cell based assay systems are particularly described that identify modulation of binding of Nrg-ICD with an Eos binding site. Other features include the promotion and/or inhibition of Nrg-ICD translocation across nuclear membranes.

10/550673

Figure 1

Human Nrg-ICD Polypeptide Sequence

SEQ	ID	NO:	
KTKKQKKLHDRLRQSLRSENNVMNMANGPHHPNPPPDNVQLVNQYVSKNIISSE		1	
RVVERETETSFSTSHYTSTTHHSMTVTQTPSHWSNGHTESILSESHSVLVSSSVENSR			
HTSPTGPRGRLNGIGGPREGNSFLRHARETPDSYRDSPH			
SERYVSAMTTPARMSPVDFHTPTSPKSPPEMSPVSSLTISIPSVAVSPFMDEERPLLL			
VTPPRLREKYDNHLQQFNSFHNNPTHE SNLPPSPLRIVEDEEYETTQEYEP AQEPPK			
KLTNSRRVKRTKPNGHISSRVEVSDTSSQSTSSSESETEDERTGEDTPFLSIQNPMATS			
LEPAAAYRLAENRTNPANRFSTPEELQARLSSVIANQDPIAV			

Figure 2

```

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/note="IG; Region: Immunoglobulin"
/db_xref="CDD:smart00409"
misc feature 1038..2222
    /gene="Nrg1"
    /note="Neuregulin; Region: Neuregulin family"
    /db_xref="CDD:pfam02158"
BASE COUNT  900 a  867 c  743 g  762 t
ORIGIN
1  gcggccgcag ctgccgggag atgcgagcgc agaccggatt gtgatcacct ttccctcttc
61  gggctgtaag agagcgagac aagccaccga agcgaggcca ctccagagcc ggcagcggag
121  ggaccggga cactagagca gctccgagcc actccagact gagcggacgc tccaggtgat
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1381  agactcctag tcacagctgg agtaatggc acacggagag cgtcatttca gaaagcaact
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1741  ctttgttga agaagagagg cctctgtctc ttgtgacgc accaaggcta cgggagaaga
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3241 accaaccaac cacaggtgcc tctgtccct gt

## Figure 3

Nuclear localization sequences

SEQ ID NO: 3                      KTKKQRKK

SEQ ID NO: 4                      PRLREKK

# Figure 4

Binding sites in Nrg-ICD for Eos

SEQ ID NO: 5      KTKKQRKKLH DRLRQSLRSE RNNVMNMANG PHHPNPPPDN  
VQLVNQYV

SEQ ID NO: 6  
SERYVSAMTTPARMSPVDFHTPTSPKSPPSEMSPPVSSLTISIPSVAVSPFMDEE  
RPLLLVTPPRLREKYDNHLQQFNSFHNNPTHESNSLPPSPLRIVEDEEYETTQEYEPAQ  
EPPKKLTNSRRVKRTKPNGHISSRVEVDSSTSSQSTSSESETEDEERTGEDTPFLSIQNP  
MATSLEPAAAYRLAENRTNPANRFSTPEELQARLSSVIANQDPIAV

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## Figure 5

Human Eos binding domain for DNA

SEQ ID NO: 7

LKCDVCGMVCIGPNVLMVHKRSHTGERPFHCNQCASFTQKGNLLRHIK  
LHSGEKPFCPCNYACRRRDALTGHLRTHSVSSPTVGKPYKCNYCGRSY  
KQSTLEEHKERCHNYL

101990673

## Figure 6

Human Eos binding domain for Nrg-ICD

SEQ ID NO: 8

CEHCRILFLDHVMFTIHMGCCHGFRDPFECNICGYHSQDRYEFSSHIVRGEH  
KVG

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## Figure 7

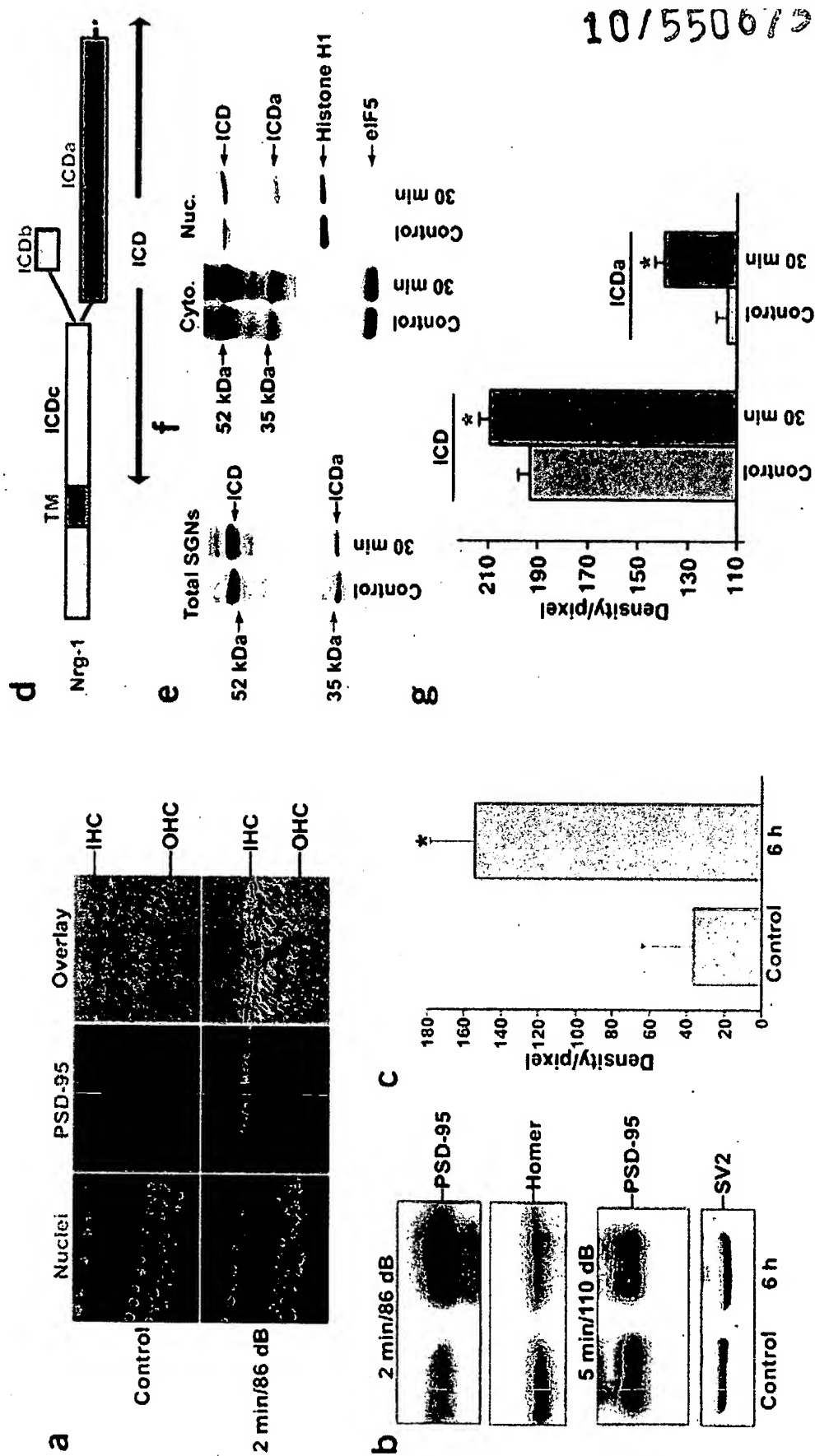
Peptides that block Nrg-ICD/Eos signaling

SEQ ID NO: 9      YGRKKRRQRRR  
CEHCRILFLDHVMFTIHMGCCHGFRDPFECNICGYHSQDRYEFSSHIVRGEHKVG

SEQ ID NO: 10      RQIKIWFQNRRMKWKK  
CEHCRILFLDHVMFTIHMGCCHGFRDPFECNICGYHSQDRYEFSSHIVRGEHKVG

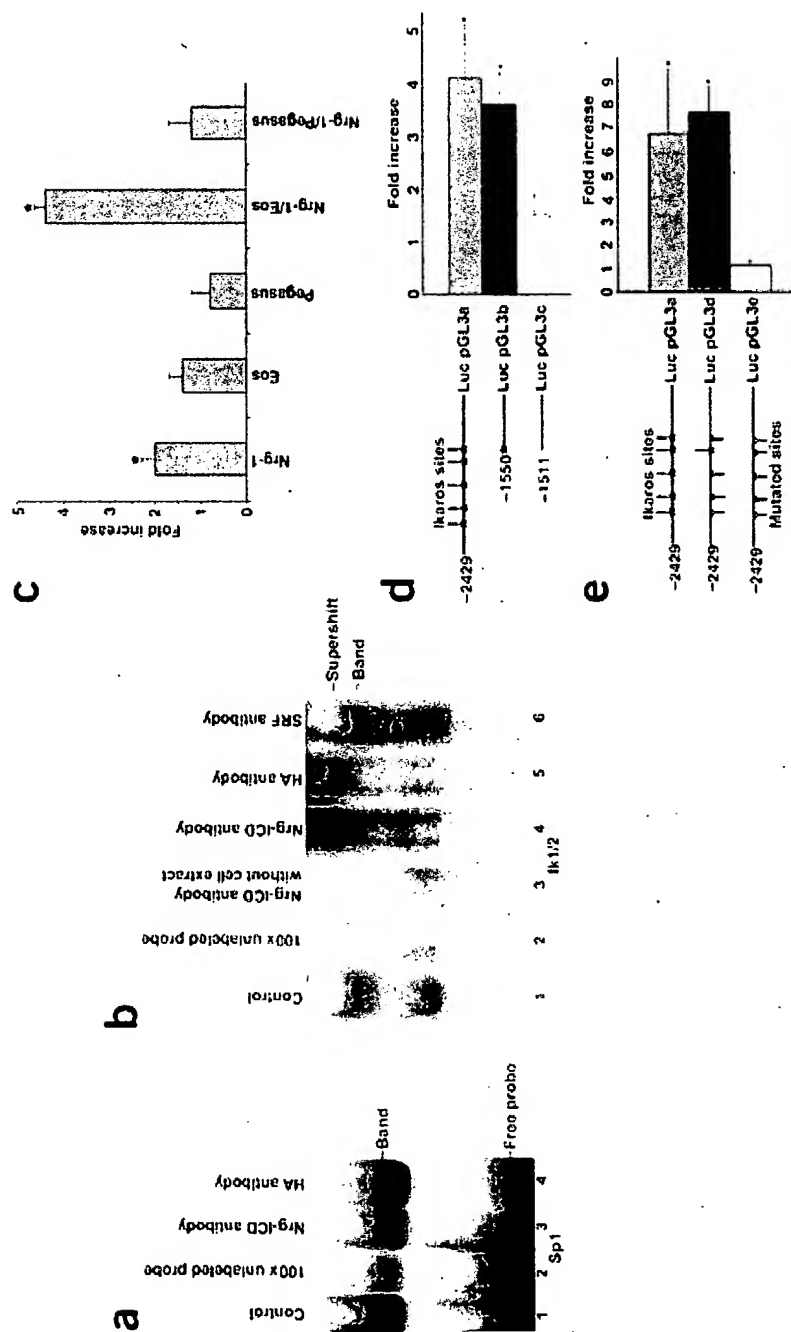
SEQ ID NO: 11      DAATATRGRSAASRPTERPRAPARSASRPRRPVE  
CEHCRILFLDHVMFTIHMGCCHGFRDPFECNICGYHSQDRYEFSSHIVRGEHKVG

Figure 8



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Figure 9

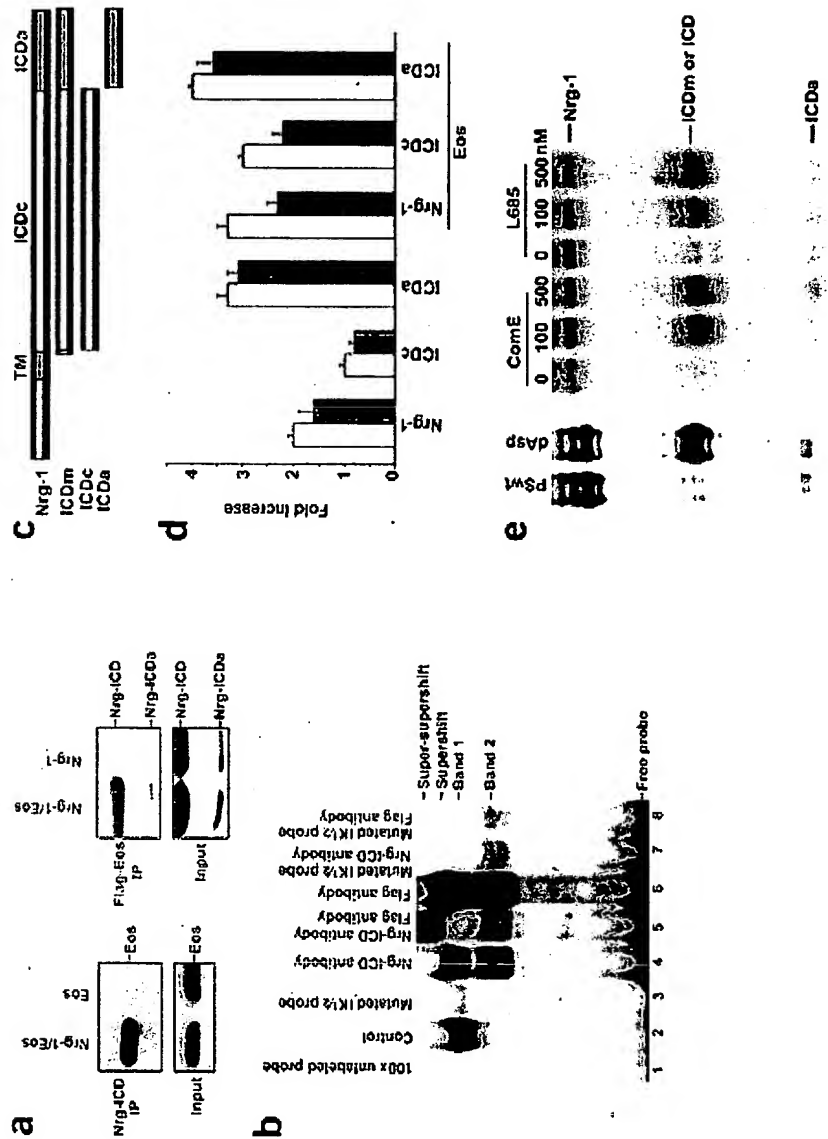


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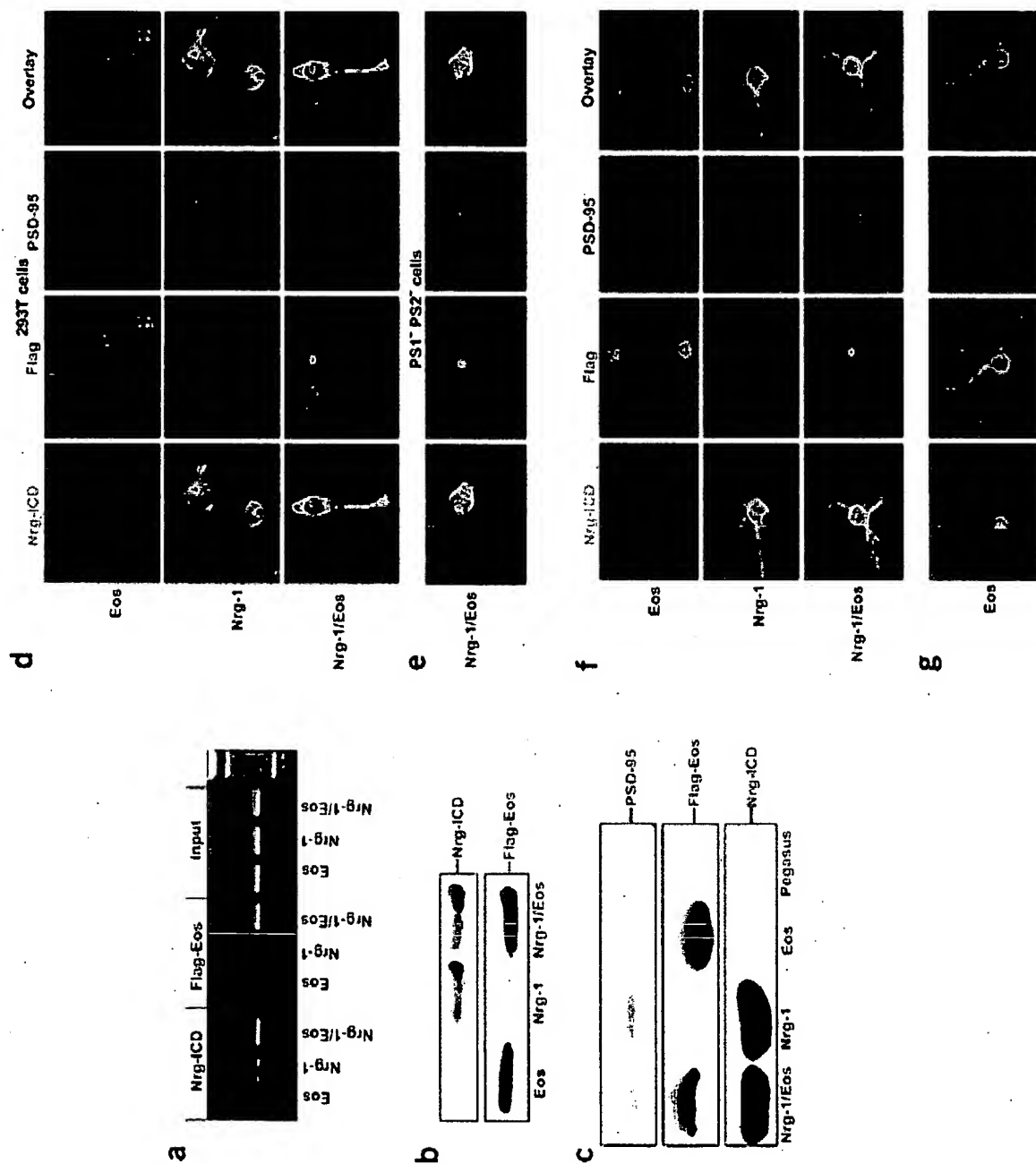


Figure 10



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Figure 11



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Figure 12

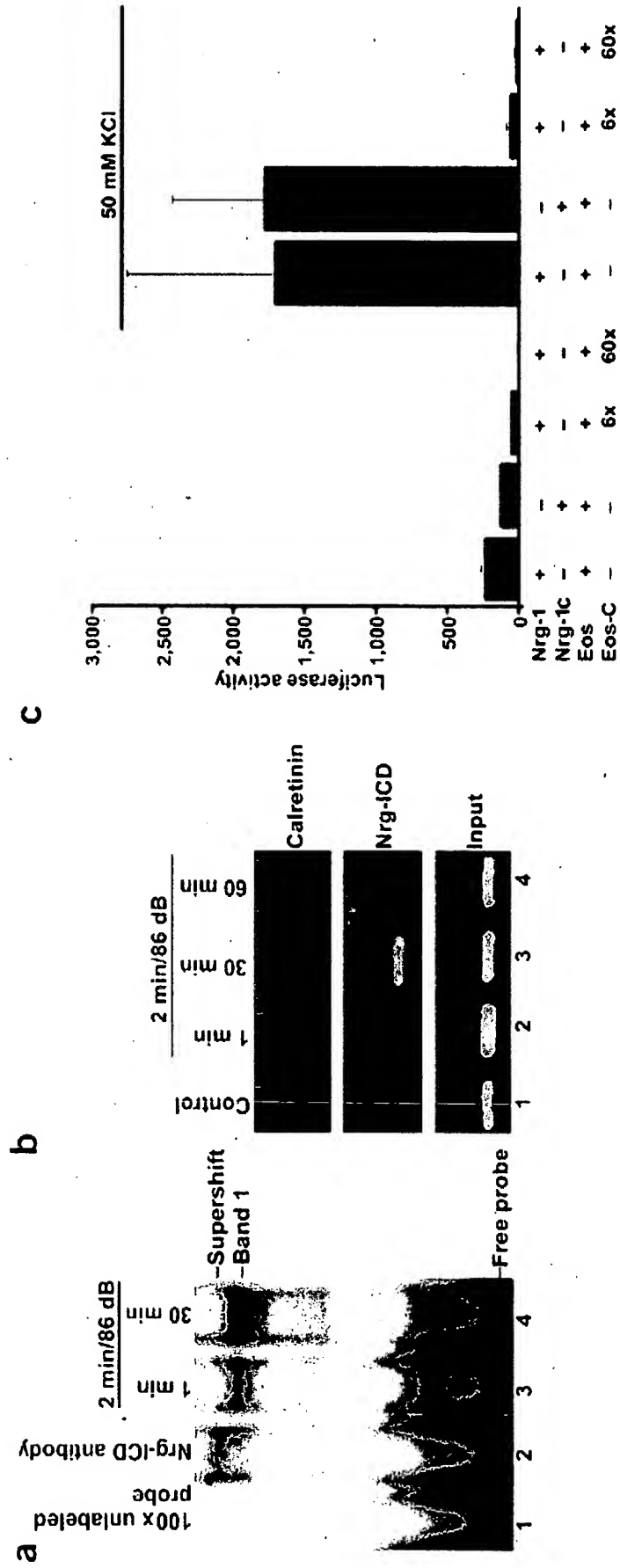
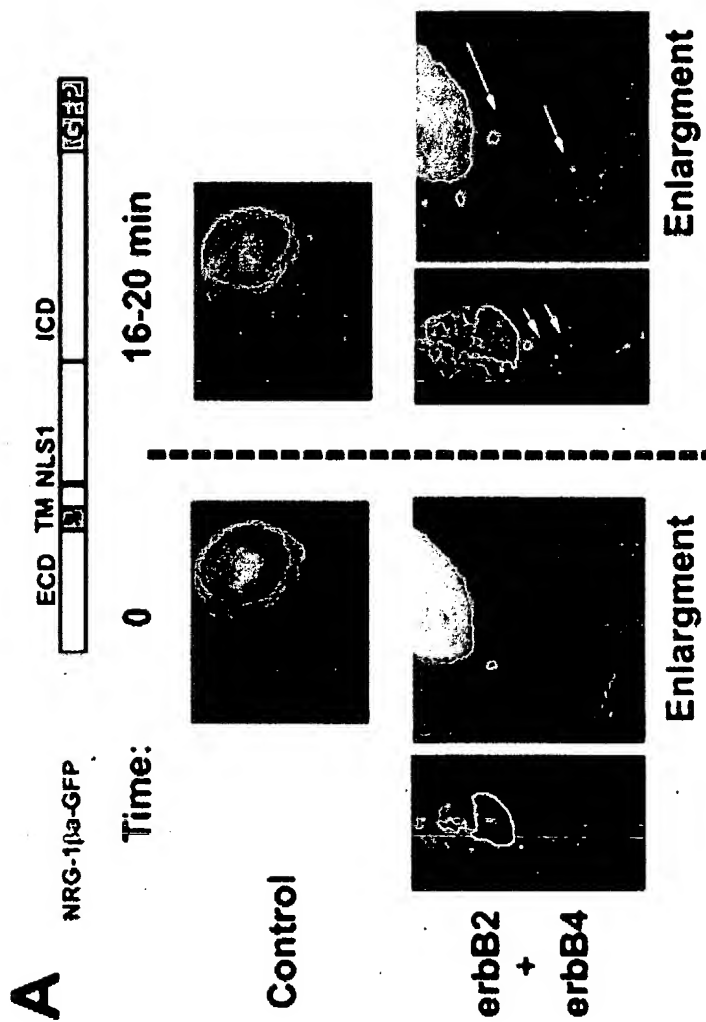


Figure 13A

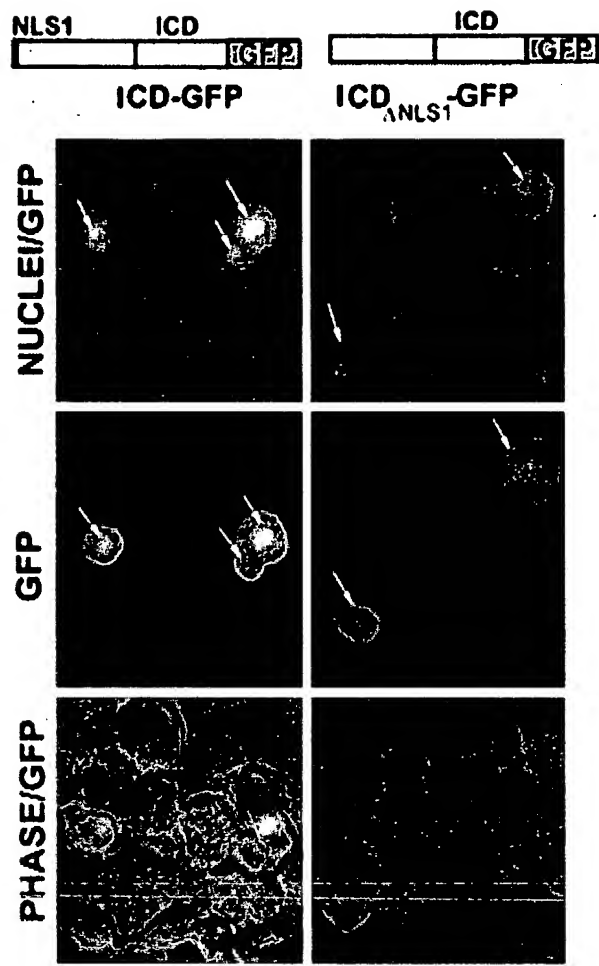


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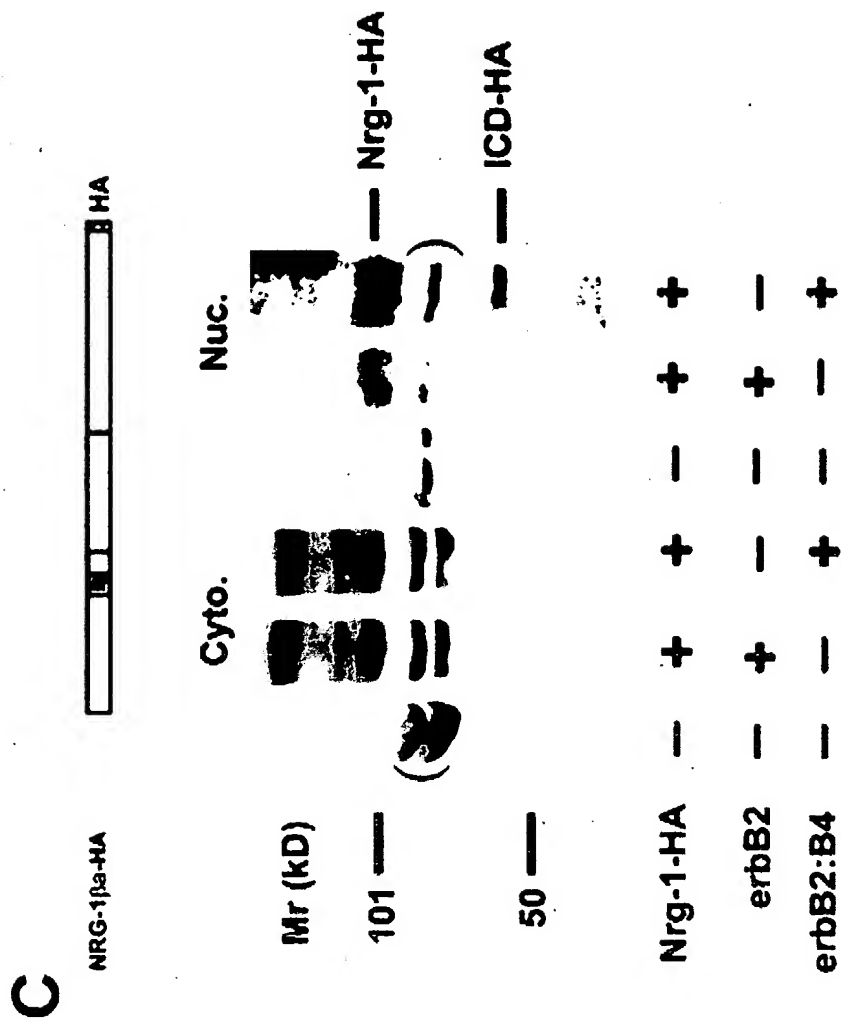
Figure 13B

**B**



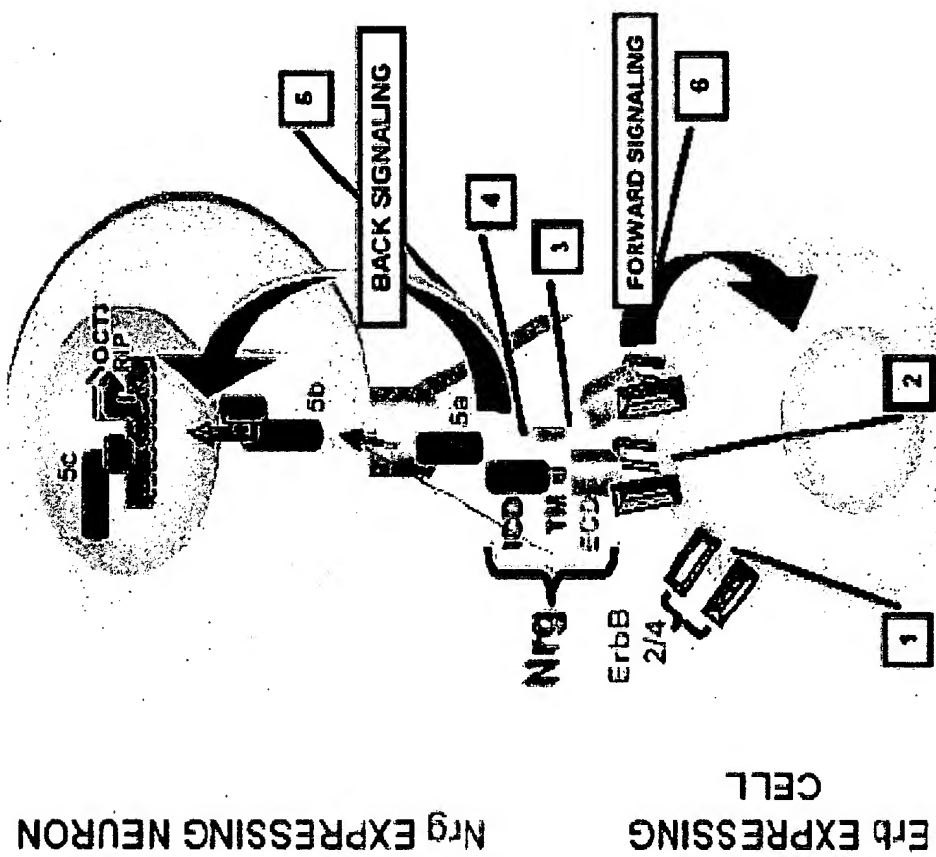
10/22/07

Figure 13C



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Figure 14



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